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GRK2 IN THE CARDIOVASCULAR DISEASE CONTINUUM: ROLE IN HYPERTENSION AND INSULIN RESISTANCE

Elisa Lucas Fernández

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ELISA LUCAS FERNÁNDEZ

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DIRECTORES DE LA TESIS:
DR. FEDERICO MAYOR MENÉNDEZ

DRA. CRISTINA MURGA MONTESINOS

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“Now, this is not the end. It is not even the beginning of the end. But it is perhaps, the end of the beginning”

Winston Churchill,
The bright gleam of victory, 1942

“It has always seemed to me extreme presumptuousness on the part of those who want to make human ability the measure of what nature can and knows how to do, since, when one comes down to it, there is not one effect in nature, no matter how small, that even the most speculative minds can fully understand.”

Galileo Galilei

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PRESENTACIÓN/PRESENTATION

En los últimos años se ha descrito el importante papel de la proteína quinasa GRK2 como nodo de señalización en diferentes tejidos y contextos celulares, y en particular como modulador de diferentes procesos y vías de señalización críticas en la fisiología cardiovascular. Los niveles de GRK2 se encuentran aumentados, tanto en humanos como en modelos experimentales animales, en procesos patológicos como la hipertensión, insuficiencia cardíaca o isquemia, mientras que la inhibición genética de GRK2 ha sido descrita como cardioprotectora en distintos modelos animales relacionados. Sin embargo, no se conocen con precisión los mecanismos moleculares por los que cambios en la expresión de GRK2 producen estos efectos deletéreos o beneficiosos. Es de destacar que GRK2 no sólo fosforila y desensibiliza diversos receptores acoplados a proteínas G (GPCR) que son críticos para la función cardiovascular tales como los receptores β-adrenérgicos o de angiotensina, sino que también modula la cascada de señalización de la insulina, muy importante para la fisiología cardiaca. Dado que estas rutas de señalización se encuentran alteradas en situaciones que son importantes factores de riesgo para el desarrollo de la enfermedad cardiovascular, como hipertensión, insulinoresistencia u obesidad, nos propusimos profundizar en las interrelaciones funcionales de GRK2 con esas vías de señalización y sus posibles implicaciones fisiopatológicas. Para ello hemos utilizado ratones hemicigotos para GRK2 (GRK2+/-) como un modelo válido para evaluar los efectos de una inhibición sistémica sostenida de GRK2 en el corazón y el tejido vascular en diferentes contextos patológicos tales como la hipertensión arterial, la edad y la obesidad/resistencia a insulina inducida por dieta rica en grasa.

Hemos observado que en ratones adultos (9-10 meses de edad, una condición más concordante con el periodo de incidencia de patologías CV en humanos) los animales GRK2+/- presentan mayor sensibilidad a algunas señales vasoconstrictoras ante determinados agonistas y son más ampliamente sensibles a diversos estímulos vasodilatadores, en parte gracias a una mayor disponibilidad de óxido nítrico incluso a nivel basal. Más aún, tras promover un estado de hipertensión primaria inducida por infusión crónica de angiotensina, los ratones con menores niveles de GRK2 preservaron la activación de la cascada de Akt, presentaron mayores niveles de la enzima eNOS, y mantuvieron una mayor biodisponibilidad de NO mientras que en sus hermanos silvestres estos parámetros disminuían significativamente. En resumen, en este trabajo demostramos que menores niveles de GRK2 favorecen una mayor biodisponibilidad de óxido nítrico por varios mecanismos, así como resistencia al desarrollo de hipertensión y del consecuente remodelado vascular.

Por otro lado, abordamos el papel de GRK2 en el corazón de ratones adultos (9 meses) o alimentados con dieta rica en grasa, dos condiciones que promueven el desarrollo de insulino-resistencia. En ratones adultos hemos observado que una menor expresión de GRK2 promueve una mayor sensibilidad cardiaca a la insulina, lo que se correlaciona con un perfil de expresión génica cardioprotector y una leve hipertrofia que no es de naturaleza patológica. Nuestro trabajo describe cómo los niveles de GRK2 aumentan en el tejido cardiaco en modelos experimentales bien establecidos de resistencia sistémica a la insulina, tales como animales alimentados con dieta alta en grasa o en los ratones obesos ob/ob. Este aumento en los niveles de GRK2 cardíacos se correlaciona con la disminución de la sensibilidad cardiaca a la insulina en estas condiciones, en un proceso que implica la formación de complejos GRK2/IRS1 que impedirían una correcta propagación intracelular de la señal de la insulina.

Por último, nuestros datos indican que menores niveles de GRK2 protegen al corazón de los efectos deletéreos de la obesidad. Los ratones GRK2+/- adultos están protegidos frente a la hipertrofia patológica de corazón que desarrollan los animales silvestres tras 33 semanas de alimentación con dieta rica en grasa, y son resistentes a la acumulación excesiva de lípidos en tejido cardiaco, característica propia de la lipotoxicidad que se produce como consecuencia de la obesidad.

Nuestros hallazgos indican que estímulos patológicos de diferente etiología, tales como elevados niveles de catecolaminas o angiotensina, o el alto contenido en grasa de la dieta, convergen en incrementar la expresión de GRK2 en corazón y en el tejido vascular. Dado el papel de esta proteína como nodo un estratégico en las redes de señalización celular tanto de GPCRs como de la insulina, este aumento de sus niveles/actividad desempeñaría un papel central en la progresión hacia la adaptación patológica, el remodelado cardiaco y vascular y la disfunción. GRK2 emerge así como un relevante nexo de unión entre la funcionalidad cardiovascular y el metabolismo, especialmente interesante en el contexto de la enfermedad cardiovascular derivada de hábitos de vida poco saludables, y, precisamente por su función nodal como sensor de múltiples patologías y como transmisor de esos efectos deletéreos, se desvela como una prometedora diana terapéutica.

Increasing evidence points to an important role for G protein-coupled receptor kinase 2 (GRK2) as a key signaling node in different tissues and cell contexts, and, in particular, as a modulator of critical processes and signaling pathways in cardiovascular physiology. GRK2 levels are increased both in humans and animal models under different pathological conditions such as hypertension, heart failure or ischemia, while the genetic inhibition of GRK2 has been described to be cardioprotective in different animal models of these diseases. However, the molecular mechanisms by which altering GRK2 expression promotes both the deleterious and beneficial effects are not completely understood. Importantly, GRK2 not only phosphorylates and desensitizes G protein coupled receptors (GPCRs) that are critical for cardiovascular function such as the β-adrenergic receptors or angiotensin, but also modulates other pathways relevant for cardiac physiology, such as the insulin cascade. Since these signaling pathways are often altered in pathological processes such as hypertension, insulin resistance and obesity, known risk factors in the development of cardiovascular disease, we aimed to investigate in detail the functional relationships between GRK2 and the aforementioned signaling pathways with focus on their potential physio-pathological implications. To this end we have used hemizygous GRK2 (GRK2+/-) mice as a suitable model to assess the effects of sustained systemic inhibition of GRK2 in the heart and vascular tissue under different pathological contexts such as hypertension, age and high fat diet- induced obesity/insulin resistance.

We observe that middle-aged adult mice (9-10 months old, a model that would better mimic the conditions in which CV events are first detected in humans) with lower levels of GRK2 (GRK2+/-) show increased sensitivity to certain vasoconstrictor signals, and are more generally sensitive to various vasodilator stimuli at least in part by means of an increased availability of nitric oxide (NO). Moreover, when primary hypertension was induced in these animals upon chronic angiotensin infusion, GRK2+/- mice displayed a preserved activation of the Akt cascade, higher levels of the eNOS enzyme, and preserved NO bioavailability whereas in their wild type littermates these parameters were significantly decreased. Furthermore, we find that lower levels of GRK2 promote increased NO bioavailability by different mechanisms, and resistance against the development of hypertension and subsequent vascular remodeling.

On the other hand, we addressed the role of GRK2 in the heart of adult (9 month) mice or of mice fed with high-fat diet, two conditions known to promote insulin resistance. In adult mice, we have unveiled that a lower GRK2 dosage promotes enhanced insulin sensitivity what correlates with a cardioprotective gene expression profile and a mild hypertrophy that is not of a pathological nature. Interestingly, cardiac GRK2 levels increased in well-established experimental models of systemic insulin resistance, such as after high-fat diet feeding or in the obese ob/ob mouse strain. Such increased cardiac GRK2 levels correlate with the impaired cardiac insulin sensitivity observed in these conditions, which seems to involve enhanced formation of GRK2/IRS1 complexes that prevent the proper intracellular propagation of the insulin signal.

Finally, our data indicate that lower levels of GRK2 safeguard the mouse heart from the deleterious effects of obesity. Adult GRK2+/- mice are protected against the pathological cardiac hypertrophy that develops in wild type littermates after 33 weeks of high-fat diet feeding, by preventing an excessive lipid accumulation inside the heart, a feature of lipotoxicity produced as a consequence of obesity.

Our findings indicate that pathological stimuli of different etiology, such as increased levels of catecholamines/angiotensin, or high dietary fat, would converge on promoting enhanced GRK2 expression in cardiac and vascular tissues. Given the role of this protein as a central node of both GPCRs and insulin signaling cascades, such increased GRK2 levels/functionality would play a key role in allowing progression to pathological maladaptation, cardiac and vascular remodeling and dysfunction. GRK2 thus emerges as a relevant link between cardiovascular function and metabolism, particularly interesting in the context of cardiovascular disease related to unhealthy lifestyle habits, and, given its role as a sensor of multiple pathological insults and as a transmitter of deleterious effects, as a promising therapeutic target.

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ABBREVIATIONS

AC: Adenilyl cyclase
ACh: Acetylcholine
AMPK: AMP-activated protein kinase
Ang II: Angiotensin II
AP-1: Activator protein-1
AR: Adrenergic receptor
AT1R: Angiotensin receptor 1
C/EBPβ: CCAAT/enhancer-binding protein beta
CaM: Calmodulin
Cav: Caveolin
CD36: Cluster of differentiation 36
Cdk2: Cyclin-dependent kinase 2
CHF: Congestive heart failure
CIRKO: Cardiac Insulin Receptor Knockout
CPT: Carnitine palmitoyltransferase
CRP: C-reactive protein
CVD: Cardiovascular disease
CVDC: Cardiovascular disease continuum
CVS: Cardiovascular system
DAG: Diacylglycerol
ECM: Extracellular matrix
EGFR: Epidermal growth factor receptor
eNOS: Endothelial Nitric oxide Synthase
Epac: Exchange protein directly activated by cAMP
ET-1: Endothelin 1
ETAR: Endothelin A receptor
FA: Fatty acid
FAO: Fatty acid oxidation
FAT: Fatty acid transporter
FFA: Free fatty acids
FOXO: Forkhead box O
G6P: Glucose 6-phosphate
GIT1: G protein-coupled receptor kinase interactor 2
GLUT: Glucose transporter

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GPCR: G protein-coupled receptor
Grb2: Growth factor receptor-bound protein 2
GRK: G protein-coupled receptor kinase
HDAC6: Histone deacetylase 6
HF: Heart failure
HFD: High fat diet
HIF-1: Hypoxia-inducible factor 1
HSL: Hormone sensitive lipase
Hsp90: Heat shock protein 90
IGF1R: Insulin-like growth factor 1 receptor
IGF-I: Insulin-like growth factor 1
IL-6: Interleukine 6
InsR: Insulin receptor
IP3: Inositol trisphosphate
IR: Insulin resistance
IRS: Insulin receptor substrate
IκBα: Inhibitor of kappa B
LV: Left ventricle
LVH: Left ventricle hypertrophy
Mdm2: Murine doble minute 2
MetS: Metabolic syndrome
MI: Myocardial infarction
MK2: MAPK-activated protein kinase 2
MLC: Myosin light chain
MLCK: Myosin light chain kinase
MLCP: Myosin light chain phosphatase
MMP: Matrix metalloprotease
mTOR: Mammalian target of rapamycin
mTORC1: Mammalian target of rapamycin complex 1
NFAT: Nuclear factor of activated T cells
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
Nppa: Natriuretic peptide A
Ox Phos: Oxidative phosphorylation
PDGFR: Platelet-derived growth factor receptor
PDK1: 3-Phosphoinositide-dependent protein kinase-1
PGC1: Peroxisome proliferator-activated receptor gamma coactivator 1

PH: Pleckstrin homology domain
Phe: Phenylalanine
Pin1: Peptidyl-prolyl cis/trans isomerase NIMA-interacting 1
PIP_2: Phosphatidylinositol 4,5-bisphosphate
PIP_3: Phosphatidylinositol 3,4,5 trisphosphate
PKA: Protein kinase A
PKC: Protein kinase C
PLC: Phospholipase C
PPAR: Peroxisome proliferator-activated receptor
RAS: Renin-angiotensin system
RAAS: Renin-angiotensin-aldosterone system
RH: Region of homology to regulators of G-protein signaling
RKIP: Raf kinase inhibitor protein
ROCK: Rho-associated protein kinase
ROS: Reactive oxygen species
SH2: Src Homology 2
SLGT: Sodium-dependent glucose cotransporter
SNS: Sympathetic nervous system
T2DM: Type 2 diabetes mellitus
T3: Triiodothyronine
TAC: Transverse aortic constriction
TCA: Tricarboxylic acid
TIMP: Tissue inhibitors of metalloproteinases
TKR: Tyrosine kinase receptor
VENIRKO: Vascular endothelial cell Insulin Receptor knockout
VSMC: Vascular smooth muscle cell
WAT: White adipose tissue
WT: Wild type

INTRODUCTION

1. THE CARDIOVASCULAR SYSTEM AND CARDIOVASCULAR DISEASE CONTINUUM

The cardiovascular or circulatory system is composed of the heart, blood vessels, and the blood. It is responsible for transporting oxygen, nutrients, hormones, and cellular waste products throughout the body to maintain homeostasis. The cardiovascular system (CVS) is essential to survival as all parts of the body require oxygen-rich blood in order to thrive. In fact, heart failure or congestive heart failure, the condition in which the heart cannot supply the body´s tissues with enough blood and the end-stage of most cardiovascular diseases (CVD), remains the major cause of death worldwide [1].

1.1. THE HEART

The primary driving force behind a healthy circulatory system is the body's hardest-working organ, the heart. Heart beats about once every second what means over 2.5 billion times in an average life span. The structure of cardiac muscle cells enables a quick propagation of electrical signals and mechanical contractions that pump blood forward. The only time the heart gets a rest is between beats [2].

The heart itself is an organ of extreme structural and functional complexity. The heart´s wall is composed of tightly packed cardiomyocytes and fibroblasts, with dense supporting vasculature and collagen-based extracellular matrix. Because of the high density and high metabolic demand of the cells, myocardium consumes large amounts of oxygen and has very low tolerance to hypoxia [3]. Cardiomyocytes are a highly differentiated cell type with very limited replicative potential after birth, thereafter subsequent growth of the heart is achieved predominantly by hypertrophy of individual cardiomyocytes. Following significant injury, the heart undergoes induced compensation and gradually deteriorates towards heart failure. Current therapy slows but does not arrest the resultant adverse remodeling and this is the reason why the best treatment for CVDs is prevention [4].

1.2. VASCULATURE

Blood pumped by the heart reaches every cell of the body through the massive network conformed by arteries, veins, and capillaries. Inside the CVS, the network of coronary arteries, cardiac veins and capillaries that irrigate the heart is of special interest because its correct function is critical to maintain a working heart and an appropriate vascular tone [5]. The largest blood vessels, such as the aorta (which is a conductance artery), have a thick, tough wall of connective tissue and many layers of smooth muscle cells. The wall is lined by a thin single sheet of endothelial cells called the endothelium, separated from the surrounding outer layers by a basal lamina. The amounts of connective tissue and smooth muscle in the vessel wall vary according to the vessel's diameter and function. In the thinnest branches of the vascular tree the walls consist of nothing but endothelial cells and a basal lamina, together with a few scattered pericytes. As the diameter of the artery narrows, as for example in mesenteric resistance arteries (MRA), the contraction and relaxation of the muscle changes vessel diameter, which alters resistance to blood flow to adapt to blood pressure fluctuations. Therefore they are referred as resistance arteries or

arterioles. In this process endothelial cells sense the shear stress due to flow of blood; by signaling this information to the surrounding connective tissue and smooth muscle cells, they enable the blood vessel to adapt its diameter and wall thickness to suit the blood flow. Endothelial cells also mediate rapid responses to neural signals for blood vessel dilation, by releasing the gas nitric oxide (NO) to make smooth muscle relax in the vessel wall. These processes are crucial for the regulation of blood pressure [6].

1.3. THE CARDIOVASCULAR DISEASE CONTINUUM

The cardiovascular disease continuum (CVDC) was first conceived by Dzau and Braunwald in 1991 and is a sequence of events precipitated by several risk factors which, if left untreated, inexorably culminate in end stage heart failure (HF) and death [7]. Despite recent improvements in therapy and clinical care, HF remains the leading cause of death in developed countries and it has been estimated that by 2030 more than 23 million people will die annually from CVD [1].

The major events of CVDC (atherosclerosis, coronary artery disease, myocardial infarction (MI), left ventricular hypertrophy and left ventricular dilation) usually begin as remodeling processes of the myocardium and vasculature to a range of potentially noxious hemodynamic, metabolic, and inflammatory stimuli in order to compensate or adapt to alterations in the CVS; but, when they persist, they progress to structural changes that become self-perpetuating and pathogenic (Figure 1) [8]. Treatment intervention at any stage during its course will either arrest or delay its progress but would never make it reversible, so the main focus remains on the prevention.

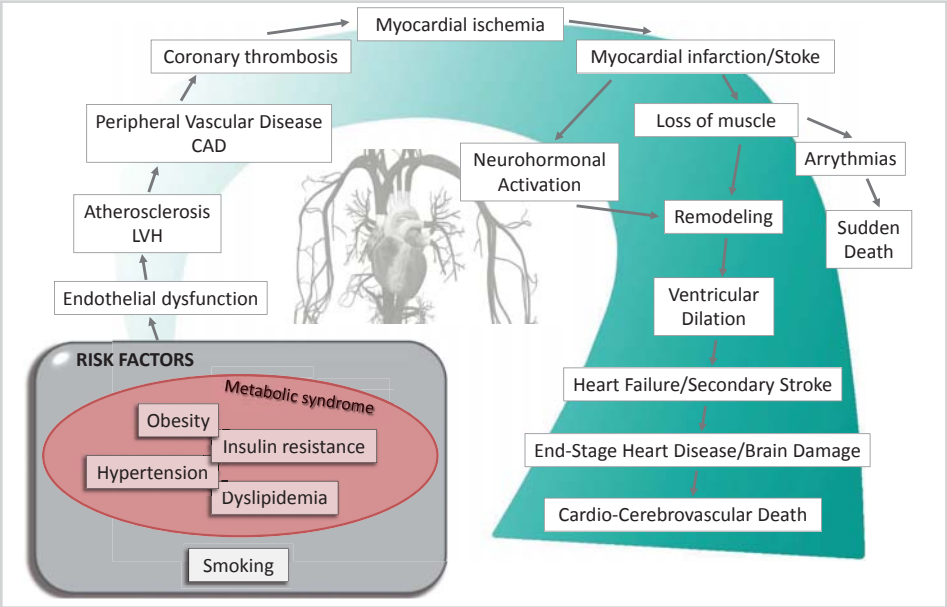

Figure 1: The Cardiovascular Disease Continuum (CVDC) is a pathological sequence of events that become progressively more severe and whose end-stage is heart failure and death. The CVDC links various risk factors that, with the exception of smoking, constitute the metabolic syndrome. By treating pathological processes that occur early on in the CVDC, like hypertension, insulin resistance or obesity it may be possible to prevent or slow the development of heart disease and to prolong life. LVH, left ventricle hypertrophy; CAD, Coronary artery disease.

2. CARDIOVASCULAR RISK FACTORS

The major risk factors of CVD are dyslipidemia, hypertension, insulin resistance (IR), obesity and smoking [9]. With the exception of smoking, all other risk factors of CVD constitute what we nowadays define as Metabolic Syndrome (MetS). MetS, also known as Syndrome X, is dramatically increasing in the wake of urbanization, surplus energy intake, increasing obesity, and sedentary life habits. The International Diabetes Federation estimates that one-quarter of the world´s adult population has the MetS [10]. It is defined by a constellation of interconnected physiological, biochemical, clinical, and metabolic factors that directly increases the risk of CVD, type 2 diabetes mellitus (T2DM), and cause mortality [11]. Further, MetS increases the risk of stroke, leads to a 3 to 4-fold increase in the risk of MI, and to a 2-fold increase in the risk of dying from such an event [12]. The physiological and biochemical effects brought about by MetS include abnormal adipose tissue functions, hepatic steatosis, heart diseases, systemic inflammation and chronic diabetes complications [13]. Therefore IR, visceral adiposity, atherogenic dyslipidemia, endothelial dysfunction, genetic susceptibility, elevated blood pressure, hypercoagulable state, and chronic stress are the several factors which constitute the syndrome [14].

2.1. OBESITY

Overweight and obesity are defined as abnormal or excessive fat accumulation that represents a risk to health [15]. The “obesity epidemic” is principally driven by an increased consumption of cheap, calorie-dense food and reduced physical activity. Adipose tissue can respond rapidly and dynamically to alterations in nutrient excess through adipocyte´s hypertrophy and hyperplasia [16]. Obesity does not only result in the storage of excessive fat by adipocytes, but rather it also constitutes a metabolic and low intensity inflammatory disorder where adipose tissue works as a hormone-generating tissue, secreting various peptides and second messengers, as well as inflammatory cytokines.

Many of these compounds (Angiotensin II (Ang II), plasminogen activator inhibitor-1, TNF-α, interleukin-6 (IL-6), C-reactive protein (CRP) and fibrinogen) are important markers in human CVDC, and correlate with the degree of obesity [17]. An imbalance between inflammatory responses associated with obesity can result in endothelial dysfunction as an initiating event in atherosclerosis [18]. Besides, the development of obesity triggers different adaptations in the morphology of cardiac structure and function. The connection between heart failure and obesity implicates complex pathophysiological mechanisms: an increase in total blood volume, and thus increased cardiac output, left ventricular hypertrophy, left ventricular systolic and diastolic dysfunction and fat accumulation. The latter was found to be in positive correlation with the degree of obesity since the myocardium gradually accumulates fat between muscle fibers what leads to myocyte degeneration and alterations of electrical conduction [19-21].

More important in the pathogenesis of obesity is that excessive body weight, besides constituting an independent risk factor for CVD, also contributes to other risk factors such as type 2 diabetes mellitus (T2DM), hypertension and dyslipidemia, which further increase the prevalence and severity of CVD [22].

BOX 1: ANIMAL MODELS OF OBESITY

Animal models with generalized obesity are frequently classified in two groups: genetically-engineered animals with mutations that result in obesity, and genetically intact animals exposed to obesogenic environments such as being maintained on high-fat diets. In evaluating results from animal models of obesity it is important to bear in mind that, as so happens in humans, obesity in certain animal models is associated with coincident morbidities such as impaired glucose tolerance, diabetes and hypertension.

Commonly used genetically-induced obese animal models are those who develop obesity because of mutations in the leptin receptor or leptin gene. One such example is the ob/ob mice, which are deficient for the leptin gene. On the C57BL6/J background they develop obesity, hyperinsulinemia, and impaired glucose tolerance shortly after weaning, and develop overt diabetes between 10 and 15 weeks of age [23] while mice with mutations in the leptin receptor (db/db) develop diabetes as early as at 5 weeks of age [24]. It is important in these animal models (ob/ob, db/db, Zucker diabetic fatty rats) to take into consideration the age at which the studies are performed, given the potentially confusing effects of hyperglycemia on the observed phenotypes. Moreover, it is important to determine which changes in these models are a consequence of obesity versus the effects that are secondary to loss of the leptin-mediated system.

Most instances of human obesity are nonetheless considered to be multifactorial, resulting from the integrated activity of numerous conditions and/or genes responsible only for a small risk factor on its own, so diet-induced obesity animals are believed to better mimic the state of common obesity in humans than most of the genetically-modified models and may be the best choice for testing prospective therapeutics [25]. Nevertheless, the pathological characteristics of animal models whose phenotype depend on the diet are also dependent on different facts such as differences in dietary lipid composition (saturated versus unsaturated fats), whether or not the high fat diets are isocaloric or promote obesity, and the period along which the animals are fed with the specific diet. Thus, C57BL6 mice fed with a high fat diet (HFD) of 55% calories from fat for 20 weeks develop cardiac dysfunction associated with mild hyperglycemia, hyperleptinemia and reduced adiponectin levels [26], although *in vivo* changes in cardiac function in mouse models with lesser degrees of obesity are more subtle. In sum, it is important to know the characteristics of each experimental animal model in order to choose the most appropriate one for each study, to analyze the results obtained and to compare them with other studies.

2.2. INSULIN RESISTANCE

Insulin-resistant individuals show an abnormal response to a glucose challenge, an elevated fasting glucose levels and/or overt hyperglycemia, or a reduction in insulin action after intravenous administration of insulin with decreased insulin-mediated glucose clearance and/or a lesser ability to suppress endogenous glucose production. An inability of the pancreatic β cells to produce sufficient insulin to correct the deteriorating IR in peripheral tissues leads to hyperglycemia and overt T2DM [27].

Type 2 diabetes (formerly called non-insulin-dependent or adult-onset diabetes) is caused by the body's ineffective use of insulin and often results from excess body weight

and physical inactivity [28]. Diabetes mellitus, especially type 2, accounts for > 97% of adult diabetic population and its prevalence has increased globally. Nowadays 382 million people are diabetic and by 2030 the number is expected to rise to 552 million reaching the 7th position in the list of leading causes of death [29].

Overt T2DM and its main feature, IR, are serious CVD risk factors and are considered a "cardiovascular risk equivalent", thus conferring diabetic patients the same risk for future cardiovascular complications as those who have already suffered a prior MI [30]. IR is responsible for diverse cardiovascular complications such as increased atherosclerosis in large arteries and increase coronary atherosclerosis promoting coronary artery disease. Besides, it can also affect cardiac structure and function in the absence of changes in blood pressure and coronary artery disease, a condition called diabetic cardiomyopathy [31]. Together, these events, acting through a variety of mediators such as altered intracellular calcium, increased reactive oxygen species (ROS), activation of the Renin-Angiotensin System (RAS), altered substrate metabolism, mitochondrial dysfunction, ceramides, hexosamines, advanced glycation end products, and more, contribute to the pathogenesis of the disorder [31, 32].

From all the above, CVD is the most common cause of death in people with IR the underlying reason being the coexistence of other well-known risk factors such as hypertension, dyslipidemia and obesity in these patients.

BOX 2: ANIMAL MODELS OF INSULIN RESISTANCE

Typical animal models of T2DM (db/db mice, ob/ob mice or Zucker fatty rats) reproduce the functional and structural alterations observed in patients with diabetic cardiomyopathy such as diastolic and systolic dysfunction and left ventricular hypertrophy (LVH) [31] however it is important to bear in mind that, as T2DM develops at varying tempos in these models, studies performed in animals before the onset of diabetes may reflect changes that are promoted by the underlying obesity and IR while studies performed after overt T2DM may reflect the added effects of hyperglycemia.

Apart from the typical animal models for the study of IR, that also constitute models of obesity and overt T2DM, engineered animal models with tissue-specific deletion of the insulin receptor enable us to investigate the specific role of impaired insulin signaling in the apparent absence of the confounding effects of systemic metabolic perturbations. Some examples of this category are CIRKO (Cardiomyocyte-specific Insulin Receptor Knock Out) mice [33] and VENIRKO (Vascular Endothelial cell Insulin receptor Knock Out) [34] mice. They are a potent tool to study the mechanistic features of insulin signaling in these specific cellular contexts but they do not fully mimic physiological IR since a total absence of the receptor is achieved instead of alterations in the finely-tuned regulation of the insulin signaling pathway observed under IR conditions. On the other hand, they do not reproduce overt IR since insulin signaling deletion is not taking place systemically; therefore they lack systemic alterations in metabolism such as hyperglycemia and hyperlipidemia, which represent highly deleterious features for CVS.

2.3. HYPERTENSION

High blood pressure or hypertension is one of the most common chronic diseases in the human population, affecting one in three adults worldwide in 2013 [35]. Hypertension has a substantial impact on public health because of its complications including kidney disease, stroke and HF (as end-stage of cardiac remodeling, LVH and ventricular dysfunction) which are major sources of morbidity and mortality.

Determinants of blood pressure are approximated by Ohm´s law modified for fluid dynamics (pressure = flow x resistance). Blood flow depends on cardiac output and blood volume, whereas resistance is determined by the contractile state of small arteries and arterioles throughout the body. Despite the physiological complexity of hypertension, only a few key pathways have a major influence on the control of blood pressure: sodium excretion by the kidney, activity of the central nervous system (SNS), and contractile processes in the vasculature [36]. The latter regulates the radius of blood vessels, thus modulating peripheral resistance and is controlled by a delicate balance of vasoconstrictor and vasodilator inputs explained below in more detail.

An important modulator of hemodynamic homeostasis is the RAS. Upon a decrease in blood flow, kidney´s juxtaglomerular cells activate their pro-renin and secrete renin directly into circulation. Renin then carries out the enzymatic conversion of liver-secreted angiotensinogen to Angiotensin I and subsequently Angiotensin I is converted to Ang II by the angiotensin-converting enzyme found in the lungs. Ang II is a potent vasoactive peptide that causes sympathetic activation, tubular water retention, arteriolar vasoconstriction and it also promotes the release of aldosterone, an hormone which fuels renal sodium reabsorption and an increase in blood pressure. Moreover the RAS can also be activated by a low concentration of salt in the kidney or by sympathetic activation [37].

An association between hypertension and inflammation has been clearly demonstrated; however, it is presently unclear whether inflammation is predominately a cause or an effect of hypertension [38]. Essential hypertension is frequently associated with several metabolic abnormalities, among which obesity and IR are the most common [39].

BOX 3: ANIMAL MODELS OF HYPERTENSION

In order to understand the pathogenesis and to study the treatment and prevention of hypertension several widely used genetic models have been generated, principally in rats, through selective breeding for the traits of increased blood pressure, salt sensitivity or both. There are also alternative animal models in which hypertension is induced by pharmacological and transgenic modulation of key neurohormonal regulators of blood pressure [36]. They share many features in common with human hypertension, as most of them have been developed based on the etiological factors that are responsible for human hypertension (such as excessive salt intake, hyperactivity of RAS, or defined genetic factors), however, these models do not fully recapitulate all the features of human hypertension. Therefore, a thorough understanding of the animal models and a rigorous analysis is required before extrapolating the findings in animals to humans.

Ang II is the final effector of the RAS, plays a major role in the physiological control of vasomotor tone and the vessel structure, and is an important contributor to blood pressure increase, vascular inflammation and fibrosis in pathological conditions such as hypertension [40]. Ang II-induced hypertension has therefore become a widely used experimental model of hypertension in rodents by its continuous administration through osmotic minipumps. It is usually delivered subcutaneously and, in the first days of the experimental model, hypertension predominantly results from the vasoconstrictor effects of the peptide, involving smooth muscle cell contraction and cytoeskeletal remodeling. In addition Ang II induces oxidative stress, which further aggravates the hypertensive phenotype and causes the release of aldosterone leading to salt and water retention resulting in increased blood volume and tension [41]. Experimental models based on Ang II infusions are reported to replicate human pathologies such as postmenopausal hypertension, preeclampsia, vascular remodeling, vascular aging, and neovascularization [42].

2.4. ENDOTHELIAL DYSFUNCTION

Another important feature of MetS that is a risk factor for CVD and is intimately related to hypertension and IR is endothelial dysfunction. It is one potential mechanism by which inflammation may promote hypertension and it is characterized by an impaired endothelium-dependent vasodilation, a reduced arterial compliance and an accelerated process of atherosclerosis [43]. Various factors like oxidative stress, hyperglycemia, advanced glycation products, free fatty acids (FFAs), inflammatory cytokines or adipokines cause an inability of endothelium to serve its normal physiological and protective mechanisms, but the key regulator in endothelial homeostasis is NO bioavailability, and IR is implicated in this process.

It is well known that IR and compensatory hyperinsulinemia, besides activating the mechanisms mentioned above, also have a toxic effect in the vasculature, mainly at the endothelial level. This takes place partly because IR impairs the production of NO, favors the production of endothelin-1 (ET-1) and the vasoconstrictive and mitogenic responses on the vascular wall [44]. Interestingly, insulin signaling pathways in vascular endothelium leading to the activation of endothelial NO synthase (eNOS) are completely independent and distinct from classical calcium-dependent mechanism used by G protein-coupled receptors, such as the muscarinic acetylcholine (ACh) receptors.

Insulin exhibits a dual and opposing action on blood vessels: NO-mediated vasodilation (via PI3K/Akt/eNOS axis) and ET-1-triggered vasoconstriction (via MAPK), so insulin induces endothelin-mediated vasoconstriction only when PI3K is inhibited as so happens in T2DM [45]. Furthermore, ET-1 promotes IR (by reducing blood supply to the skeletal muscle), increases oxidative stress and reduces the bioavailability of NO fueling a detrimental vicious circle that promotes a proatherogenic state [46]. These events take place also in genetic animal models of MetS (Zucker Obese, Spontaneously Hypertensive Obese and JCR-LA-cp corpulent rats) and in diet-induced obese animal models, not only in macrovascular but specially in microvascular resistance vessels [47].

3. CARDIAC AND VASCULAR REMODELING

Pump failure leading to congestive heart failure (CHF) is the common endpoint of the spectrum of progressive CVDs of the CVDC. Many compensatory mechanisms -such as myocardial hypertrophy and dilation, as well as neurohormonal, cytokine, and endothelial activation- precede cardiac failure; however, such myocardial (and extra-cardiac) adaptations eventually progress to a maladaptive response, and ultimately lead to decompensation and CHF. Maladaptation manifests as hemodynamic abnormalities, neurohormonal imbalance, cytokine overexpression, and endothelial dysfunction.

3.1. VASCULAR REMODELING

There is a high plasticity in vessels to accommodate changing conditions of mechanical load. The contractile state of vascular smooth muscle cells (VSMC) regulates the radius of blood vessels, thus modulating peripheral resistance.

Vascular remodeling refers to alterations in the structure and arrangement in blood vessels through cell growth, cell death, cell migration and production or degradation of the extracellular cell matrix (ECM), contributing to elevated vascular resistance in response to hypertension.

Of special interest is vascular remodeling in resistance arteries, involved in systemic vascular resistance and therefore in the regulation of arterial blood pressure as well as blood flow within tissues. These vessels are highly innervated by autonomous nerves (particularly sympathetic adrenergic), and respond to changes in nerve activity and circulating hormones by constricting or dilating.

- *Types of remodeling*

Depending on whether lumen diameter decreased or increased, remodeling could be "inward" or "outward". In resistance vessels lumen is reduced and inward remodeling occurs in primary hypertension –with RAS activation- [48] that is, of an eutrophic nature (no net change in the amount of tissue and constant cross-sectional area). However, the growth is hypertrophic (with net growth and increased cross-sectional area) in salt-dependent hypertension, T2DM and all conditions in which the endothelin system is activated. Besides, in small arteries of hypertensive rats hyperplasia of VSMC was found, whereas VSMC hypertrophy has been reported in the aorta [49].

- *Signaling pathways implicated in vascular remodeling*

Rises in blood pressure directly affect remodeling of blood vessels by increasing wall stress and stimulation of mechanoreceptors [50]. Remodeling is importantly affected by Ang II as it influences vascular structure in hypertension (Figure 2). It binds to the AT1 receptor (AT1R), leading to the transactivation of receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and insulin-like growth factor-1 receptor (IGF-1R), and nonreceptor tyrosine kinases, such as c-Src [51]. In addition, Ang II:AT1R binding induces activation of NADPH oxidase resulting in intracellular generation of ROS, which influence redox-sensitive

signaling molecules, such as mitogen-activated protein kinases (p38, JNK, ERK1/2 and ERK5), transcription factors -NF-κB, AP-1 and HIF-1- and matrix metalloproteinases (MMP) [52]. Ang II may also downregulate peroxisome proliferator-activated receptors (PPARs), which have anti-inflammatory effects, thus enhancing vascular inflammation [53]. Then, under pathological conditions, altered Ang II signaling leads to altered growth, fibrosis, and inflammation, which altogether contribute to structural remodeling in hypertension [40].

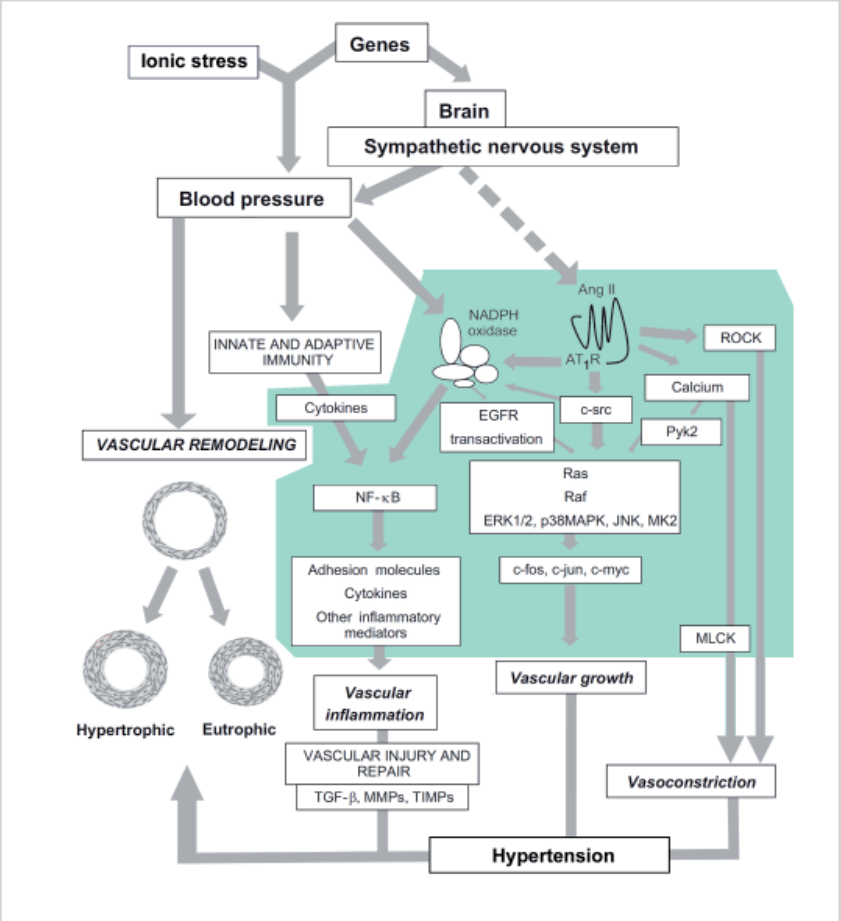

Figure 2: Mechanisms governing hypertension and vascular remodeling. In the presence of genetic predisposition and unfavorable environmental conditions (eg, excess salt or inappropriate diet), the brain through activation of the sympathetic nervous system will induce small rises in blood pressure. Blood pressure elevation directly affects remodeling of blood vessels by increasing media stress and stimulation of mechanoreceptors. It may also stimulate oxidative stress in the vascular wall by enhancing NADPH oxidase activity. Remodeling of the wall is importantly affected by angiotensin II (Ang II), which stimulates calcium release leading to vasoconstriction. Ang II–induced vasoconstriction also acts via Rho kinase (ROCK), which increases myosin light chain (MLC) sensitivity to calcium. Besides, Ang II signaling assembles in several MAPKs signaling pathways which regulate cell growth, apoptosis, inflammation and migration. Growth, inflammation, and repair processes interact with vasoconstriction promoting hypertension which promotes vascular remodeling. EGFR indicates epidermal growth factor receptor; ERK, extracellular regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; MK2, MAPK activated protein kinase-2; MLCK, MLC kinase; MMP, matrix metalloprotease; TGF-β, transforming growth factor-β; TIMP, tissue inhibitor of MMP. Shadowed in green are the molecular mechanisms that lead to hypertension (Adapted from Schiffrin E.L. 2012).

Endothelin and aldosterone acting in an endocrine and paracrine fashion may also induce deposition of collagen and fibronectin with increased collagen:elastin ratio in small vessels thus promoting an enhanced stiffness of the vasculature [54, 55].

3.2. CARDIAC REMODELING

3.2.1. Cardiac hypertrophy

The heart has a unique ability to adapt to acute as well as chronic changes in the hemodynamic state in order to maintain optimal circulation. Thus, the process of cardiac hypertrophy is an adaptive phenomena initiated in response to an increased hemodynamic overload.

Cardiac remodeling is defined as alterations in size, geometry, shape, composition and function of the heart resulting from cardiac load or injury. Left ventricular (LV) remodeling is frequently seen in hypertensive subjects and has been considered an adaptive response to hemodynamic overload imposed by systemic hypertension. This compensatory response is assumed to be explained by the Laplace´s law, T =P x r/2h, where tension or stress in the LV wall (T) is directly related to LV pressure (P) and radius (r) and is inversely related to LV wall thickness (h). Therefore, sustained elevated blood pressure leads to increases in LV wall stress [56].

- *Types of remodeling*

In response to increased LV wall stress, LV wall thickens and LV mass increases, thus resulting in normalization of wall stress and the development of a structural pattern known as concentric hypertrophy (see Figure 3). Alternatively, increases in blood volume would lead to an increase in the chamber radius, resulting in eccentric hypertrophy [57]. Apart from this etiologic/morphometric classification, cardiac hypertrophy can be further categorized as “compensated” or “decompensated” based on LV chamber ejection performance and the presumed stage of the deteriorating condition, but these terms are often used to describe the presence or absence of cardiac failure, regardless of whether cardiomyocyte function is normal or abnormal. Cardiac hypertrophy could also be classified as “physiological” or “pathological”. The former refers to a temporary increase in the size of the organ due to a physiological demand such as during normal postnatal maturation or, in adults, during pregnancy in females, or with chronic exercise training. In these cases, cardiomyocytes grow longitudinally and no changes in expression of fetal genes or collagen are detected [58]. This type of hypertrophy is reversible and it is also called “adaptive”. However, under prolonged pathological stress, such unremitting hypertension [57] and therefore increase pressure overload instead of volume overload, T2DM [59] or obesity [60], cardiac hypertrophy is accompanied by interstitial fibrosis, contractile dysfunction, altered gene expression pattern with re-expression of fetal genes, changes in energy metabolism with diminished fatty acid oxidation and abnormal electrophysiological properties which eventually lead to decreased systolic and diastolic function and overt decompensated heart failure [61]. This kind of LV hypertrophy is considered of a pathological nature and in fact constitutes as an independent risk factor for cardiovascular morbidity and mortality [62].

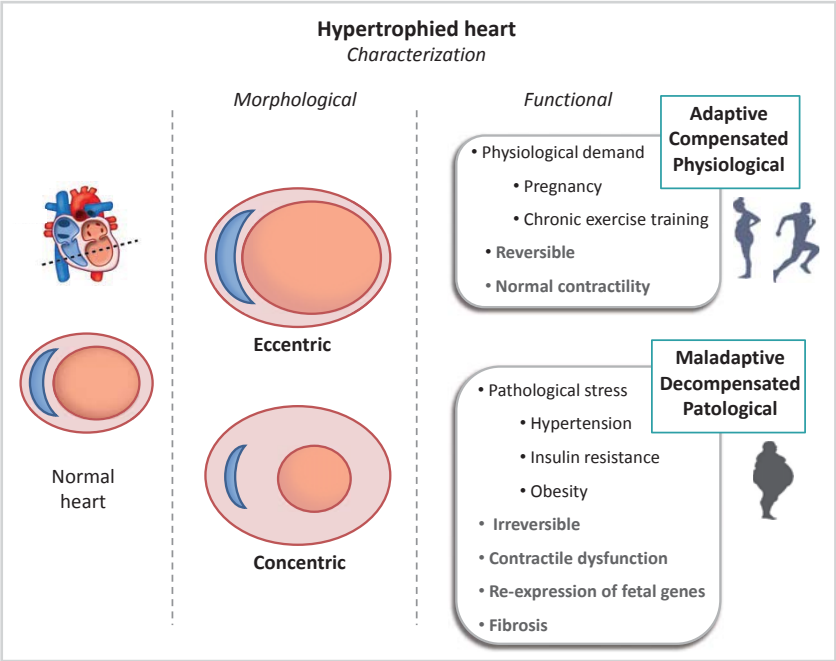

Figure 3: Types of cardiac hypertrophy could be classified from a morphological point of view in eccentric or concentric if the radius of the ventricles is increased or diminished respectively, or from a functional point of view, taking into account the final outcome in heart function and morphometry: whether contractility is affected or not, or if the remodeling is permanent or reversible.

Cardiac remodeling could evolve to diastolic and systolic dysfunction. Echocardiographic measurements are very useful to evaluate cardiac functionality and characterize cardiac hypertrophy. Also, fractional shortening and ejection fraction are two prototypical measures of cardiac function. The first is a simple measure of the pump function of the heart as the ratio between the diameter of the left ventricle when it is relaxed and its diameter when it has contracted. The ejection fraction indicates the fraction of blood in the ventricles pumped out with each heartbeat expressed in percentual terms. Heart failure risk varies with LV geometric pattern, with eccentric and concentric hypertrophy predisposing to HF with reduced and preserved ejection fraction, respectively.

Whereas in hypertension- and obesity-mediated LV hypertrophy the most frequent geometric pattern is eccentric hypertrophy, either concentric or eccentric remodeling was reported in diabetic patients. Nevertheless, the coexistence of hypertension with either T2DM or obesity increases the proportion of concentric hypertrophy in patients [57, 60]. Interestingly, although under pathological conditions subjects with concentric hypertrophy are predisposed to the highest cardiovascular risk [63], physiological concentric hypertrophy can also be developed under resistance training [64].

- *Signaling pathways implicated in cardiac hypertrophy*

The hypertrophic growth of the myocardium is typically initiated by signal transduction pathways in response to either neuroendocrine factors or an ill-defined mechanical stretch or wall tension-sensing apparatus [65].

Physiological hypertrophy is initiated by finite signals, which include growth hormones (such as thyroid hormone, insulin, insulin-like growth factor 1 (IGF-1) and

vascular endothelial growth factor (VEGF), and mechanical forces that converge on a limited number of intracellular signaling pathways (such as PI3K, Akt, AMPK and mTOR). These pathways or effectors have been shown to antagonize cell death in the heart or to stimulate myocyte renewal, suggesting that physiological growth stimulation through such pathways can be cardioprotective despite causing mild heart enlargement [66]. On the other hand, pathological hypertrophy is promoted by higher levels of neuro-endocrine hormones such as ET- 1 and Ang II secreted under biomechanical stress. Signaling pathways in pathological hypertrophy converge in the $G\alpha_q$/PLC/Ca^{2+}/PKC and calcineurin/NFAT axis. Other examples of mediators of pathological cardiac hypertrophy are the p38 and JNK branches of the MAPK cascade, which phosphorylate and activate the GATA4 transcription factor. Catecholamines can also signal to adenylyl cyclase (AC) to induce activation of protein kinase A (PKA), which then phosphorylates an array of intracellular targets, leading to increased calcium release and enhanced contractility, the net effect being cardiomyopathy as a result of increased myocyte apoptosis and necrosis [65].

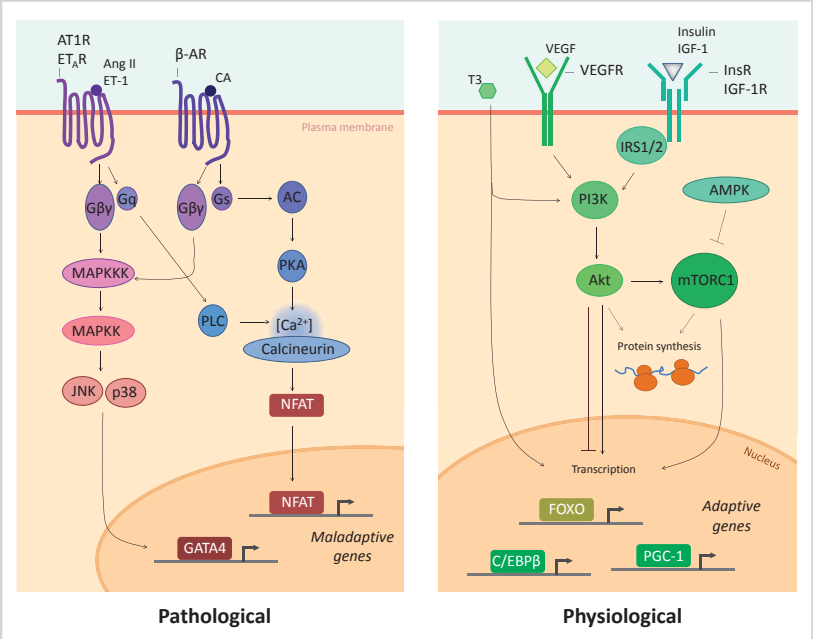

Figure 4: Signaling pathways implicated in pathological and physiological heart hypertrophy. In the first case disorders such as systemic hypertension, myocardial infarction or metabolic cardiomyopaty, among others, are responsible for the activation of $G\alpha_s$- and $G\alpha_{q/11}$-coupled receptors such as β-adrenergic or angiotensin receptos (AT1R). Calcium–calcineurin–NFAT pathway downstream $G\alpha_{q/11}$/AC/PKA and $G\alpha_s$/PLC are related to cardiomyocytes growth and the pathological hypertrophy of the heart. On the other hand the p38 and JNK branches of the MAPK cascade phosphorylate and activate the GATA4 transcription factor. Both NFAT and GATA4 transactivate the transcription of hypertrophic target genes that are typically maladaptive. The net effect of this appears to be cardiomyopathic, as it results in increased myocyte apoptosis and necrosis. On the other hand, physiological hypertrophy is initiated by intermittent signals of triiodothyronine (T3), vascular endothelial growth factor (VEGF), insulin and insulin-like growth factor 1 (IGF1). Downstream their receptors we can find common signalling branches controlled by PI3K, Akt and mTOR complex 1 (mTORC1), whereas AMP-activated protein kinase (AMPK) governs metabolic adaptive reprogramming. These signalling pathways regulate the transcription of adaptive genes, protein synthesis, metabolism and energy production through a set of transcription factors such as FOXO, PGC1 or C/EBPβ among others. AC indicates adenylyl cyclase; PKA, protein kinase A; PLC, phospholipase C.

3.2.2. Metabolic remodeling of the heart

The heart is a constitutive energy-demanding organ. It must contract incessantly, thus the requirement for energy to fuel optimal function is immense. Daily turnover of cardiac ATP is calculated to range between 6 and 35 kg and its storage within the cardiomyocytes is minimal, only sufficient to sustain the heart beat for a few seconds, so a tight coupling between ATP production and myocardial contraction is essential for normal cardiac function [67].

The heart is capable of using all classes of energy substrates, including carbohydrates, lipids, amino acids, and ketone bodies for ATP production in the mitochondria [68, 69].

It is widely accepted that fatty acids are the predominant substrate used in the adult heart; however, the cardiac metabolic network is highly flexible in using other substrates when they become abundantly available [70].

Under pathological conditions metabolic flexibility is impaired and a shift takes place in myocardial substrate utilization that limits the potential of adaptation of the heart to supply ATP for continuous cardiac contraction.

3.2.2.a) Changes in cardiac metabolism with pathological hypertrophy and failure

Cardiac metabolism undergoes a reprogramming in response to pathological hypertrophy, characterized by increase dependency on glucose metabolism and decreased fatty acid oxidation (FAO) what implies a reversion to the fetal energy substrate preference pattern [71]. This shift in substrate preferences is associated with the downregulation of the transcriptional mechanisms for FAO and mitochondrial biogenesis mediated via PPARα and PGC1α [72].

Metabolic remodeling in the hypertrophied heart is associated with decreases in the overall ATP synthesis by oxidative metabolism because, although glycolysis is increased, its contribution to total ATP synthesis is limited as glycolytic ATP accounts for less than 5% of the total energy used by the heart [73]. Nevertheless, a shift from FAO to glucose could be considered beneficial for HF caused by chronic ischemic cardiomyopathy, since it improves oxygen efficiency for ATP synthesis in a condition where oxygen supply is limited [74].

Another aspect to consider is that HF may itself promote metabolic changes such as IR in part through neurohumoral activation. The failing heart invokes compensatory SNS and RAS activation, which increase blood FFAs thereby promoting IR and vasoconstriction in peripheral tissues which may lead to endothelial dysfunction perpetuating the vicious cycle: HF → metabolic alterations → HF [67].

3.2.2.b) Metabolic cardiomyopathy associated with obesity and diabetes mellitus: Glucotoxicity and Lipotoxicity

Cardiomyocytes are capable of metabolizing a spectrum of substrates which are, however, unable to enter the cardiomyocyte by simple diffusion and must be taken up by facilitated transport. Fatty acid uptake is mediated by FAT (fatty acid translocase, also known as cluster of differentiation 36, CD36), and glucose intake is accomplished by GLUT4 (glucose transporter type 4). In response to availability of nutrients or increased cardiac work, plasma insulin concentrations rise [75]. This, in turn, provokes GLUT4 as well as FAT/CD36 translocation to the myocyte sarcolemma. During the development of IR and T2DM, FAT/CD36 becomes preferentially sarcolemma-localized, whereas GLUT4 is internalized. This reciprocal positioning of GLUT4 and FAT/CD36 is central to aberrant substrate uptake in the diabetic heart, where fatty acid metabolism is chronically increased at the expense of glucose [76].

In obese or individuals with T2DM, cardiac dysfunction observed independently of macro- and microvascular disease is considered a consequence of diabetic cardiomyopathy. It is characterized by an increased FA uptake and oxidation associated with reduced glucose oxidation, either because of an increase in FA supply through the diet, an insulin-resistant state that promotes the uptake and oxidation of FA or the combination of both (see Figure 5). Noteworthy, although FAO is the main source of ATP in cardiomyocytes, increased rates of FAO may be detrimental to cardiac function due to the increase in myocardial oxygen consumption, reduced cardiac efficiency by uncoupling of the mitochondria and increased oxidative stress [31]. Interestingly, when the supply of substrates exceeds the needs for ATP synthesis, lipids accumulate in the heart. The increase in the rate of FAO eventually leads to its own downregulation, accumulation of toxic lipid intermediates as ceramides, and contractile failure, what is known as lipotoxic cardiomyopathy [77]. The maintenance of high levels of mitochondrial β-oxidation are critical to reduce the excessive fat accumulation and storage leading to obesity; furthermore lipid overload involving triacylglycerol accumulation in non-adipose tissues characterizes disorders such as heart dysfunction both in humans and in animal models of obesity and IR [78].

On the other hand, high levels of circulating glucose (hyperglycemia) promote several cardiovascular complications as a result of cardiac and vascular tissue responses to toxic metabolites from glucose metabolism, which has been referred to as cardiac and vascular glycotoxicity. Glucose could enter in the cardiomyocyte through insulin-independent glucose transporter GLUT1 or Na^+-dependent glucose transporter (SGLT) 1 and 2 [79]. Indeed SGLT1 expression has been found increased in the cardiomyocytes of ob/ob mice [80], and because of the shift towards FAO, glycolitic intermediates fuel alternative metabolic pathways with deleterious effects to the cardiomyocytes. Multiple molecular mechanisms have been suggested to mediate glycotoxic effects on cardiovascular tissues such as increased polyol pathway, activation of the diacylglycerol/PKC pathway, increased oxidative stress, overproduction and action of advanced glycation end-products, and increased hexosamine pathway. In addition, the alterations of signal transduction pathways induced by hyperglycemia or toxic metabolites can also lead to cellular

dysfunctions and damage vascular tissues by altering gene expression and protein function. Hyperglycemia might also inhibit some endogenous cardiovascular protective factors such as insulin, VEGF and PDGF, which play important roles in maintaining vascular homeostasis [81].

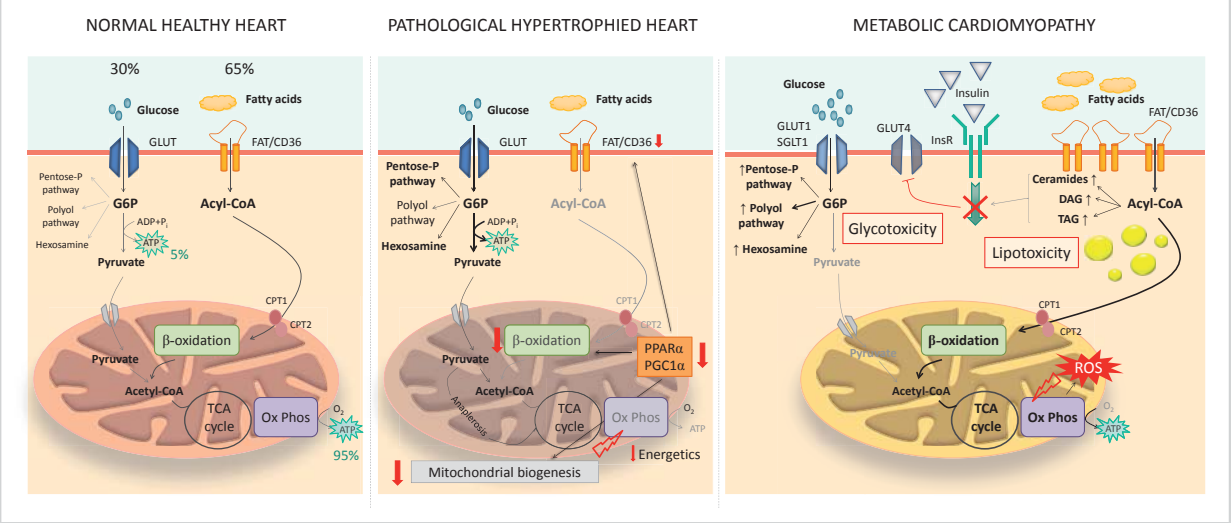

Figure 5: Metabolic remodeling of the heart under different pathological conditions. A healthy heart, under a balanced nutrient disposal, obtained its energy as ATP preferentially from fatty acid oxidation. It accounts for the 65% of total ATP whereas glucose metabolism represents 30%. The pathologically hypertrophied heart is characterized by the downregulation of the transcription factor PPARα and the transcriptional cofactor PGC1α what downmodulates fatty acid uptake and catabolism. On the other hand, glucose metabolism is reduced to glycolisis, what limits ATP synthesis, and fuels accessory pathways that result in the production of metabolites that are of important biological function although do not directly contribute to energy supply. Therefore the hypertrophied heart is considered an energy-starved model with high risk of suffering heart failure. On the contrary, in the metabolic cardiomyopathy characteristic of obese and/or insulin-resistant individuals, the abrogation of insulin signaling is the main feature, and is enhanced upon excessive dietary fat. The cardiomyocyte increases fatty acid uptake and oxidation leading to increased oxygen consumption and ROS production, what impairs mitochondrial function. An excessive flux of fatty acids to the cell promotes the accumulation of toxic intracellular fatty acid species in a process known as lipotoxicity. Also, hyperglycemia promotes glycotoxicity fueling alternative metabolic pathways for glucose that generate increased redox stress, O-glycosilated proteins and so on, leading to alterations in vascular and cardiac structure and function. G6P indicates Glucose-6-phospate; TCA cycle, tricarboxylic acid cycle; CPT, Carnitine palmitoyltransferase; Ox Phos, oxidative phosphorylation; InsR, insulin receptor.

4. MOLECULAR SIGNALING PATHWAYS IN CVS PHYSIOLOGY

4.1. GPCR SIGNALING

G protein-coupled receptors (GPCRs) are a conserved family of seven transmembrane-spanning receptors that constitutes the largest class of receptors targeted by medicinal drugs [82]. Ligand binding induces a conformational change in the GPCR, which triggers coupling with heterotrimeric guanine-nucleotide regulatory proteins (G proteins). The four primary families of Gα-proteins ($G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$) diverge with respect to downstream signaling molecules and subsequent physiological responses (see Figure 6).

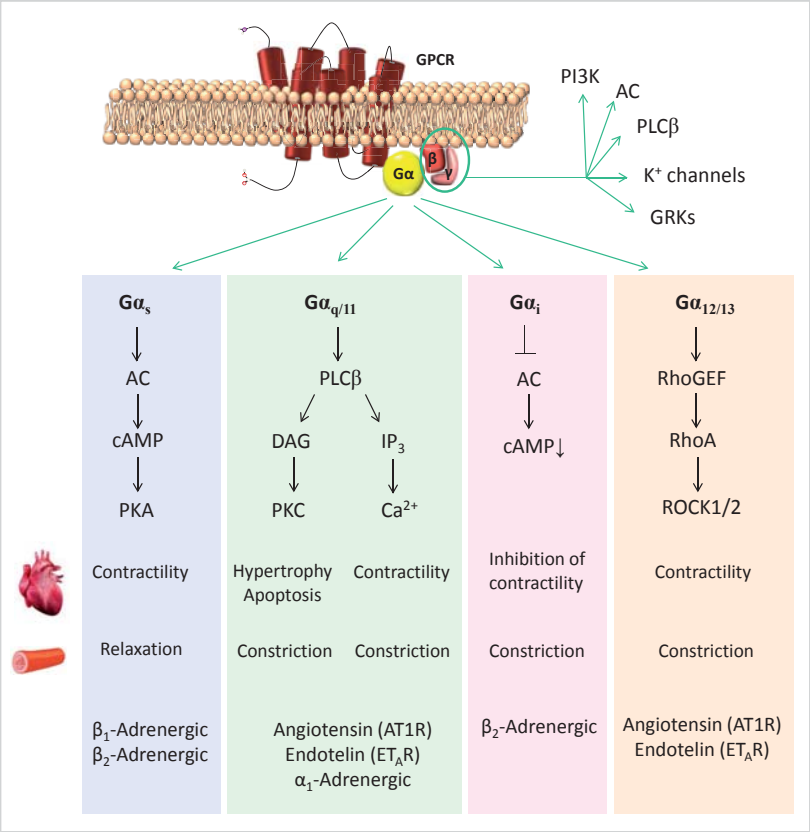

Figure 6: GPCRs in the cardiovascular system. The main characteristics of GPCR signaling are represented related to the Gα protein to which they are coupled. In each case its main effects in both heart and vessels and some important GPCRs are highlighted. AC indicates adenylyl cyclase; PLCβ, phospholipase C β; cAMP, cyclic adenosine monophosphate; RhoGEF, guanine nucleotide exchange factors for Rho GTPase; DAG, diacylglycerol; IP_3, Inositol trisphosphate; PKA, protein kinase A; PKC, protein kinase C.

Upon ligand binding, the activated GPCR interact with the G protein and serves as a guanine-nucleotide exchange factor to promote GDP dissociation and GTP binding to Gα. This results in the activation of the heterotrimeric G protein and the dissociation of the Gα from the Gβγ subunits to mediate downstream signaling [83]. Gβγ subunits signal via effectors such as PI3K, AC, PLC, potassium channels, and GRKs [84]. Endocytosis of GPCRs leads to their removal from the plasma membrane, after which they are sorted to either lysosomes for degradation or to recycling endosomes for subsequent reinsertion into the plasma membrane. GRK-dependent recruitment of β-arrestins to the phosphorylated receptor is crucial for clathrin-dependent endocytosis, which is the predominant internalization pathway described for the majority of GPCRs. Interestingly, some receptors are regulated by a single GRK, whereas others appear to be regulated by many GRKs. In fact, recent reports suggest that distinct phosphorylation patterns by different GRKs establish a signaling barcode that determines β-arrestin functionality [85, 86]. Desensitization of GPCRs can also be initiated by their phosphorylation by second messenger-dependent kinases such as PKA and PKC, that can be activated by other receptors, an so called heterologous desensitization, in contrast to GPCR desensitization by GRKs in which only agonist-bound receptors are desensitized and is referred to as homologous desensitization [82].

4.1.1. GPCRs and cardiac function

Among the estimated 200 cardiac GPCRs, drugs targeting adrenergic and angiotensin GPCR signaling pathways alone account for the majority of prescriptions for CVD treatments.

Adrenergic receptors connect the sympathetic nervous and CVS, playing an integral role in the rapid regulation of myocardial function. In response to the reduced cardiac output of the failing heart, the SNS releases neurohormones to both activate cardiac pumping and retain salt and water [87]. However, in heart failure, chronic catecholamine stimulation of ARs may contribute to pathologic cardiac remodeling, including myocyte apoptosis and hypertrophy [65]. Cardiac myocytes express at least six types of ARs, including three types of β-ARs (β_1, β_2, and β_3) and three types of α_1ARs (α_{1A}, α_{1B}, and α_{1D}) [88].

Systemic release of catecholamines (epinephrine and norepinephrine) stimulates β-ARs of the myocardium, increasing heart rate, enhancing contraction, and improving cardiac performance [83]. However sustained β-AR stimulation can be deleterious with time, causing receptor desensitization and downregulation due to, among other factors, increased levels of GRK2. The resulting loss of responsiveness to catecholamines fuels contractile dysfunction [89]. β_1-ARs couple primarily to $G\alpha_s$ proteins to regulate heart rate and contractility [83]. However, during heart failure, the β_1-adrenergic receptor subtype is downregulated in response to increased catecholamine stimulation. Chronic activation of this receptor results with time in myocyte apoptosis, cardiac hypertrophy and decreased contractility [88, 90]. β_2-ARs couple to G_s proteins, but can also switch to G_i-coupling after receptor phosphorylation by PKA [91]. β_2-AR stimulation initially activates G_s signaling pathways in a pattern analogous to β_1-AR activation. This stimulatory pathway is quickly overshadowed by the delayed activation of the G_i pathway, which negatively regulates contractility in response to prolonged β-AR activation [92]. In the healthy heart, β_1- and β_2-adrenergic receptor subtypes are expressed at a ratio of 70:30 [93]. This ratio changes in the failing heart reaching 60:40 what contributes to HF [94].

β_2-ARs are also important for mediating smooth muscle relaxation and appear to have an anti-apoptotic effect in cardiomyocytes mediated by the Gβγ subunits of G_i which activate the PI3K/Akt axis [88]. Other proteins such as the AMPK, mTOR, and ERK1/2 have recently been suggested to be targets of β_2-ARs [95]. Finally, β_3-AR have been described to mediate negative inotropic effects in cardiac myocytes [96] and differ from the other two subtypes in: i) their resistance to homologous desensitization [97] (they are not phosphorylated by GRK2); ii) their upregulation in pathological conditions, such as ischemic or dilated cardiomyopathies in humans; and iii) in their quantity, being the less abundant subtype in the heart -although they have been detected in microvascular endothelial cells and cardiac myocytes from normal and diseased human hearts [98]-. Experimental studies have shown β_3-AR couple to G_s and G_i proteins [99].

Angiotensin II receptors (AT1R) are upregulated in the ischemic and hypertrophic states of the diseased heart [100], and downregulated during end-stage HF [101]. Ang II engages signal transduction pathways that differ between cell types. In cardiac fibroblasts Ang II activates MAP kinases through a pathway including the Gβγ subunits of G_i, Src, Shc, Grb2, and Ras, while G_q and PKC are activated in cardiac myocytes [102]. Similar to AT1R the expression of endothelin-1 receptor (ET_A) is upregulated in the diseased heart and its chronic activation in this condition promotes hypertrophy and pathological remodeling

[103]. Importantly, the overactivation of these and other G_q-coupled receptors have deleterious effects to the cardiac tissue. Furthermore, cardiac overexpression of G_q-coupled receptors or $G\alpha_q$ itself constituvely active in transgenic mice leads to myocardial hypertrophy [104, 105], apoptosis and heart failure [104, 106]. On the contrary, α_1-ARs that are also G_q-coupled receptors mediate important protective and adaptive functions in the heart [107] however they are only a minor fraction of total cardiac ARs. Interestingly chronic activation of β-AR and α_1-AR stimulates cardiac myocyte hypertrophy in animals and in cultured cells [90] and both are involved in norepinephrine-induced MAP kinase activation.

4.1.2. GPCRs and vascular function

Regional blood flow can be efficiently regulated at the local level by the intrinsic ability of vessels to respond to various mechanical forces such as wall tension and shear stress but also to chemical triggers such as metabolites and local oxygen pressure. While endothelium regulates local vascular tone by a delicate balance in the release of vasoactive molecules, the VSMCs are mainly regulated by neural control mechanisms, the renin–angiotensin pathway and the vasopressin pathway that interact closely to maintain normal levels of blood pressure [37].

Numerous GPCR agonists -including Ang II, endothelin, norepinephrine, and epinephrine- provide the neurohormonal inputs that modulate vascular smooth muscle contraction and blood pressure. More specifically, vasoconstriction through Ang II, endothelin, and α_1-adrenergic receptor activation is counteracted by β-AR mediated vasodilation, finely-tuning vascular tone.

Typical vasoconstrictor receptors such as AT1R, ET_AR and α_1-ARs are $G_{q/11}$-coupled and enhance intracellular Ca^{2+} concentration leading to myosin light chain kinase (MLCK) activation. Increased intracellular Ca^{2+} levels are not only due to IP_3-mediated Ca^{2+} release from the sarcoplasmic reticulum, but also to Ca^{2+} influx through cation channels or voltage-gated Ca^{2+} channels. In addition, many $G_{q/11}$-coupled receptors have been shown to activate RhoA, thereby contributing to Ca^{2+}-independent smooth muscle contraction [108].

Most of the known physiological effects of Ang II are mediated by AT1Rs, which are widely distributed in all organs. AT1R has two isoforms that are functionally and pharmacologically indistinguishable [109] however in vivo experiments show that the $AT1_AR$ isoform may be more important than $AT1_BR$ in regulation of blood pressure [110]. Even though most of the vasoactive effects of Ang II occur via AT1Rs, AT2Rs have been shown to exert anti-proliferative and pro-apoptotic changes in VSMCs, mainly by antagonizing AT1Rs. AT2R expression declines after birth, thus may play an important role in fetal development, and can be induced later in adult life under pathological conditions. Interestingly, AT2R does not internalize upon agonist binding [111].

Similarly, endothelin, produced primarily in the vascular endothelium, signals through two GPCRs, ET_A and ET_B promoting different effects in the vessels. The ET_A receptor is

expressed predominantly at the surface of VSMCs, where it mediates proliferation and smooth muscle contraction. It is coupled to $G_{q/11}$ but also to G_s and $G_{12/13}$ leading to stimulation of AC, PLC/PKC pathway and small RhoA [112]. On the other hand ET_B exerts an important role in the clearance of ET, release of nitric oxide and prostacyclins from endothelial cells which affect endothelial cell survival, the inhibition of endothelin converting enzyme and vasorelaxation [113]. The ET_B has two subtypes, ET_{B1} which is at the surface of endothelial cells and responsible of the effects of endothelin over this cell type, and ET_{B2} which is expressed in smooth muscle cells and is coupled to G_i and $G_{q/11}$ thus implicated in the contraction of this cells.

Vascular smooth muscle relaxation is mediated by a variety of mechanisms, one of them being direct activation of G_s-coupled receptors like the adenosine A_2 or β_2-adrenergic receptors on the surface of smooth muscle cells. Another key mechanism in regulating vascular smooth muscle tone is the nitric oxide (NO)-cGMP system. Vascular endothelial cells normally produce NO, which diffuses to adjacent VSMCs where it activates soluble guanylate cyclase leading to increased formation of cGMP and vasodilation. cGMP can activate a cGMP-dependent protein kinase, inhibit calcium entry into the vascular smooth muscle, activate K^+ channels, and decrease IP_3 [114]. NO effects depend, among others, on NO concentration, compartmentation of NOS enzymes and local redox conditions of cells and tissues [115].

Nevertheless the production of NO in endothelial cells may also be governed by GPCRs on the endothelial surface such as B_2-bradykinin receptor or acetylcholine M_3-muscarinic receptor. Their stimulation activate intracellular pathways such as IP_3/Ca^{2+}/calmodilin pathway downstream G_q, that modulate eNOS activity post-translationally through heat shock protein 90 (Hsp90) or Akt-phosphorylation [115].

Both neurotransmitters and hormones released from the autonomous nervous system cooperate to preserve the balance between vasoconstriction and vasorelaxation and to control cardiac muscle cells function, and it is now generally accepted that NO exerts a critical role in this context in the CVS [116].

4.2. INSULIN SIGNALING

A substantial body of evidence has reported that insulin has direct actions on the CVS independent of its systemic effects on plasma glucose or lipids. The specific binding of insulin to its cognate cell surface receptor initiates activation of complex signal transduction networks that regulate diverse cellular functions [117].

Then, upon insulin binding to its receptor, a ligand-activated tyrosine kinase, the phosphorylation of intracellular substrates takes place including IR substrate (IRS) family members and Shc that serve as docking proteins for downstream signaling molecules. Most insulin signals are produced or modulated through tyrosine phosphorylation of IRS1 or its homolog IRS2. IRS1 is involved in the control of body growth and peripheral insulin action, whereas IRS2 helps regulate brain growth, body weight, glucose homeostasis, and female fertility [118]. Tyrosine phosphorylation of IRS family members

at multiple sites creates Src homology 2 (SH2)-domain binding motifs for numerous SH2 domain-containing effectors including PI3K and Grb2. PI3K converts PIP_2 (an inositol phospholipid of the plasma membrane) to PIP_3 that recruits Akt and 3-phosphoinositide dependent protein kinase–1 (PDK1) to the plasma membrane where PDK1 activates Akt. In turn, Akt phosphorylates and subsequently activates many substrates responsible of the metabolic actions of insulin. It is also responsible for GLUT4 translocation to the membrane and mTOR/p70S6K activation that promote protein synthesis which is one of the signaling pathways implicated in physiological heart hypertrophy. In the endothelial cells of the vasculature, Akt also leads to the phosphorylation and subsequent activation of eNOS, promoting the production of NO [44].

In addition to PI3K-dependent insulin signaling, another major insulin signaling branch involves tyrosine-phosphorylated IRS1 or Shc binding to the SH2 domain of Grb2 that results in activation of the preassociated GTP exchange factor Sos. This activates the small GTP binding protein Ras, which then initiates a kinase phosphorylation cascade involving Raf, MEK, and ERK [119]. This MAPK-dependent branch of insulin signaling pathways generally regulates biological actions related to growth, mitogenesis, and differentiation but in endothelial cells this branch also promotes the acute expression of ET-1 [120].

In sum, the PI3K-dependent branch of insulin signaling in vascular endothelium regulates the vasodilator actions of insulin, whereas the MAPK-dependent branch tends to promote the prohypertensive actions of insulin in various tissues. Therefore under healthy conditions, the various cardiovascular actions of insulin exist in a balance that contributes to cardiovascular homeostasis and result in a minimal net hemodynamic effect on blood pressure (Figure 7).

These two major branches of insulin signaling pathways also regulate distinct biological functions related to regulation of cardiovascular homeostasis. For example, in VSMC the PI3K/Akt insulin signaling branch attenuates contractility by two mechanisms: regulating agonist-induced increases in cytosolic calcium through voltage-sensitive calcium channels, and altering the activity of myosin light chain phosphatases [121]. Altogether, these effects result in an overall decrease in vasoconstrictor tone [122].

Insulin signal transduction pathways are arranged in highly complex networks that include multiple feedback loops, cross-talk between major signaling branches, and cross-talk from signaling pathways of heterologous receptors [123]. A key feature of insulin resistance in the vessels is that it is characterized by a specific impairment of PI3K-dependent signaling pathways, whereas other insulin signaling branches including Ras/MAPK-dependent pathways are unaffected [124]. Therefore under IR conditions causing metabolic alterations, endothelial dysfunction becomes a precipitating factor in the development of CVDs.

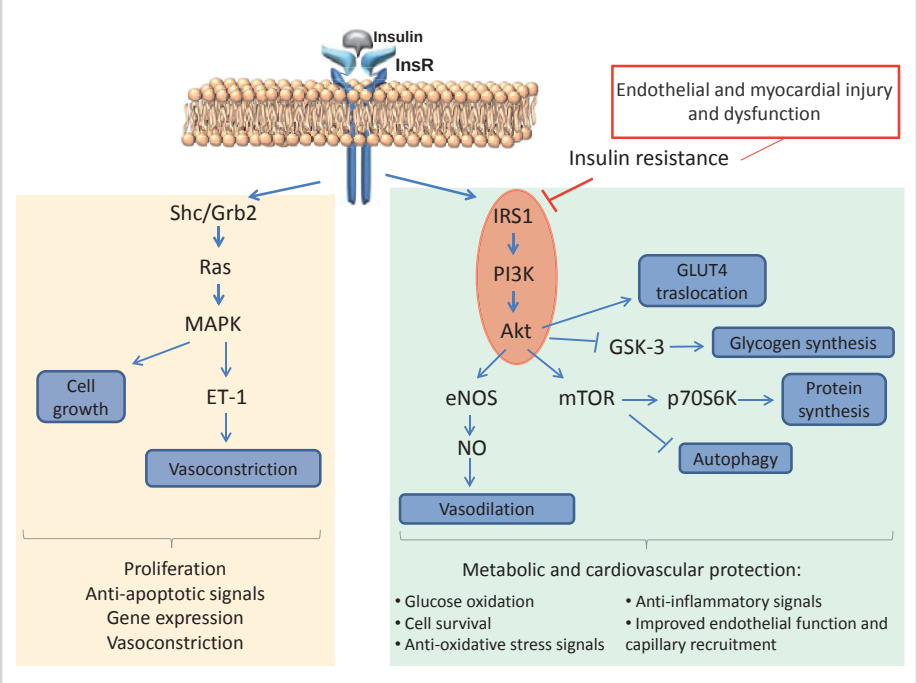

Figure 7: Insulin signaling in the cardiovascular system. General features of insulin signal transduction pathways are represented. The PI3K branch of insulin signaling regulates glucose metabolism and protein synthesis while stimulating NO production and vasodilation in vascular endothelium. The MAPK branch of insulin-signaling pathways generally regulates growth and mitogenesis and controls secretion of endothelin (ET-1) in vascular endothelium. The IRS1/PI3K/Akt axis, responsible for the metabolic and cardiovascular protection pathways, is impaired under IR conditions leading to cardiac hypertrophy, oxidative stress, apoptosis, cardiac remodeling and alterations in contractility, altogether contributing to endothelial and myocardial injury and dysfunction.

5. GRKS IN THE CARDIOVASCULAR SYSTEM

G protein-coupled receptor kinases (GRKs) are emerging as important integrative nodes in many cellular processes. They regulate numerous GPCRs by phosphorylating the intracellular domain of the active receptor, leading to the recruitment of the multifunctional adaptor proteins, arrestins, to the receptors leading to the attenuation of intracellular G protein-dependent signaling by desensitization and internalization of the receptor [125]. Moreover, emerging evidence suggests that GRK2, the most widely studied member of this family of kinases, modulates multiple cellular responses in various physiological contexts by either phosphorylating non-receptor substrates or by directly interacting with signaling molecules [126] including components of the insulin signaling pathway (*article #2*).

In particular, GRK2 emerges as a signaling hub which display a very complex interactome, whose expression and function is tightly regulated and with changes in its levels or functionality highly related to the onset or development of CVDs and/or its comorbidities [126-129].

5.1. THE G PROTEIN-COUPLED RECEPTOR KINASE (GRK) FAMILIY

GRKs are a family of serine-threonine kinases composed of seven members (GRK1-7) that share a global homology of 60-70% and are grouped in three subfamilies: GRK1 (rhodopsin kinase) and GRK7 (cone opsin kinase) are the so called visual GRKs and are present in the retina, the β-Adrenergic receptor kinase family (β-ARK) that comprise GRK2 (β-ARK 1) and GRK3 (β-ARK 2) which are ubiquitous, and a third subfamily, which includes GRK4 -present in testis, cerebellum and kidney- and GRK5 and 6 which are ubiquitous as well [89].

GRKs share structural and functional similarities: a well-conserved central catalytic domain is flanked by N-terminal domain, that, except for visual GRKs, includes a region of homology to regulators of G-protein signaling (RH) with weak homology among the members of the different subfamilies and that in the case of GRK2 and GRK3 has been shown to allow the interaction with $G\alpha_{q/11}$ proteins [130], and C-terminal domain of variable length, which have little or no sequence homology between the different GRKs. It is more variable in extension and function and determines the localization of these proteins at the plasma membrane [131, 132]. A pleckstrin homology domain (PH) in GRK2 and GRK3 mediates their agonist-induced translocation from the cytosol to the plasma membrane through interactions with phospholipids (PIP_2) and with Gβγ subunits at the membrane surface [133, 134]. On the contrary, GRK5 is predominantly associated to cellular membranes by means of basic sequences at the N-and C-terminus of the protein. GRK1, 4, 6 and 7 are constitutively anchored to the plasma membrane, visual GRKs through short C-terminal prenylation sequences and GRK4 and GRK6 through palmitoylation sites [135].

Activation of GRKs is achieved by direct interaction with Gβγ subunits [134] and also through docking to active GPCRs in a process involving allosteric interactions with receptor domains and membrane lipids which promote intracellular rearrangements and lead to increased enzymatic activity [135].

5.2. GRKs IN CVD

GRK2, 3 and 5 are important modulators of the CVS functionality. They are expressed ubiquitously but most highly in myocardial and endothelial cells [136, 137]. However only GRK2, 5 and 6 are detected in rat mesenteric smooth muscle cells [138].

The two more widely studied GRKs, as far as their impact on CVD is concerned, are GRK2 and GRK5. It has been recently reported that both are required for heart development in zebra fish with overlapping and opposing functions regulating different morphological outcomes [139]. Interestingly, increased expression and activity of any of them is associated with human and experimental models of CVD such as ischemic [140] or idiopathic dilated cardiomyopathy [137], cardiac ischemia [141], hypertension [142, 143](*article #1*), LVH [144] and HF [145-148]. Intriguingly, as members of different GRK subfamilies, they have different structural and functional characteristics, subcellular localization, and kinase activity regulation [149-151].

GRK2 is predominantly cytoplasmic and is targeted to the membrane upon agonist stimulation by interacting with free Gβγ dimers which recruit GRK2 to the agonist-bound GPCR, culminating in the phosphorylation of the receptor [134]. A mitochondrial localization for a small population of GRK2 has also been reported [151, 152] as well as an association of GRK2 to microsomal endomembranes [153]. In contrast, GRK5 lacks the PH domain, is not modulated by Gβγ, and consequently does not undergo agonist-induced membrane translocation. Instead, the carboxyl terminus of GRK5 contains a PIP_2-binding domain and basic amino acids which interact with plasma membrane phospholipids, constitutively localizing GRK5 to cell membranes. A nuclear localization for GRK5 has also been reported [149] [144, 154]. This particular feature, together with divergent receptor specificity, seems to be directly relevant to GRK5-mediated regulation of gene transcription through its role as a histone deacetylase kinase under hypertrophic stimuli what is critical for its contribution to CVD [144]. Indeed, although they share certain characteristics, GRK2 and GRK5 are non-redundant enzymes with specific functional and regulatory properties. For example, GRKs 2 and 5 attenuate cardiac contractile responses to β-AR stimulation, but only GRK2 affects Ang II receptor-mediated contraction [155, 156]. Besides, phosphorylation of the Ang II receptor by GRK2 modulates β-arrestin-mediated GPCR internalization, whereas phosphorylation by GRK5 regulates β-arrestin-dependent signalling to MAPK [157]. There are 4 polymorphisms described for human GRK5, one of which substitutes the amino acid leucine for glutamine at position 41 (in close proximity to a regulatory domain at the N-terminus) and leads to enhanced desensitization of β_2-AR upon agonist treatment [158], but no relevant mutations or SNPs have been described so far for GRK2 in humans. This fact, together with the embryonic lethality of the GRK2 knockout (GRK2 KO) mice, may reflect its overall importance in physiology.

In sum, GRKs expressed in the CVS are not functionally redundant, but rather serve distinct physiological purposes that coincide with their unique structural and substrate properties.

5.3. GRK2 AS A SIGNALING NODE IN CVD

5.3.1. Importance of GRK2 in the CVS

Gene ablation of GRK2 is embryonic lethal by day 10.5 [159]. A number of cardiac abnormalities have been described in GRK2 knockout embryos, including myocardial hypoplasia, chamber dilation, and diminished ventricular contraction as assessed by intravital echocardiography [159, 160]. However, because the heart is the first organ to form in the developing embryo, cardiac abnormalities are common even in embryonic lethal mouse models that do not have a primary cardiac defect [161]. In fact cardiac-specific GRK2 KO or GRK2 hemizygous mice developed normally and exhibited no adult cardiac phenotype at baseline other than modestly enhanced contractile function [160] what demonstrated that GRK2 has a broader extra-cardiac role in embryogenesis. Consistently, GRK2 KO mice have marked vascular malformations involving impaired recruitment of mural cells [162]. Also, GRK2 regulates cell cycle in different ways [163,

164] and Smoothened-dependent proliferation and patterning during embryonic development [126, 165]. Altogether, these evidences lead to the conclusion that GRK2 is a relevant signaling hub able to regulate several important cellular processes during embryogenesis.

The overall importance of GRK2 to the CVS has been demonstrated through several studies with genetically engineered mice. The global loss of GRK2 (GRK2-/-) by homologous recombination leads to embryonic lethality with cardiac malformations and dysplasia [159], however the conditional cardiac specific GRK2 KO mice developed normally [160]. On the other hand, in a conditional cardiac GRK2 ablation mouse model in which cardiac myocyte GRK2 expression was normal during embryonic development, but was ablated after birth (αMHC-Cre × GRK2fl/fl), improved function and prevention of HF development was observed after MI. Moreover, when the downregulation of GRK2 is induced in the cardiomyocytes after HF development by tamoxifen administration (αMHC-MerCreMer×GRK2fl/fl), there is an active reverse remodeling and improved cardiac function [166]. On the contrary, cardiac-specific GRK2 overexpressing mice display a loss of β-AR-mediated inotropic reserve, as well as desensitized Ang II receptors (AT1R) in their hearts [155].

To study the contribution of GRK2 to different cellular processes of CVD, animal models that overexpress a carboxy-terminal peptide of GRK2 (termed βARKct since GRK2 was previously called β-adrenergic receptor kinase) have been extensively used. βARKct overexpression seems to act by inhibiting endogenous GRK2 activation by competing with Gβγ binding. Transgenic βARKct mice have increased function at baseline and in response to the β-AR agonist isoproterenol [155], but this improved performance causes no myocardial damage. In fact overexpression of βARKct rescued several mouse models of HF such as after LV pressure overload induced by TAC (transverse aortic constriction) [167] or after acute myocardial ischemia/reperfusion injury [168]. Furthermore the cross breeding of βARKct mice with different animal models of chronic HF induced by genetic manipulation also rescued overt cardiac failure in these animals [146, 147].

Other important animal model for the study of the role of GRK2 in the CVS is that for endothelial cell-specific GRK2-downregulation using a Tie-2-Cre (Tie2Cre-GRK2fl/fl), which resulted in abnormalities and changes in vasculogenesis since GRK2 is deleted prenatally in this murine model [162].

Despite the variety of animal models used to study the role of GRK2 in CVS, we have chosen hemizygous GRK2 mice (GRK2+/-) for our work. Heterozygous GRK2 knockout mice with circa 50% less GRK2 in all tissues show increased cardiac function and responses to adrenergic input [146], present increased energy expenditure due to a more functional BAT tissue [169] and are also more sensitive to insulin and resistant to age, TNF-α or HFD-induced IR, obesity and adiposity [170]. This model represents, in our view, the best model to study the consequences that an overall decrease in GRK2, such as the one that could be obtained upon treatment with a yet to be identified selective GRK2 inhibitor, can have specially in cardiovascular perturbations derived from metabolic alterations (see below).

5.3.2. Regulation of GRK2 function in the CVS

5.3.2.a) Control of GRK2 function

While, quantitatively, the most important activators of GRK2 are GPCR intracellular domains and free Gβγ subunits [171], several proteins important for cardiac physiopathology are also able to associate with GRK2 and appear to control GRK2 activity and determine the complex subcellular distribution of the kinase such as α-actinin [172], clathrin [173], calmodulin [174, 175], caveolin [130] or RKIP [176].

Phosphorylation of GRK2 is also emerging as a key mechanism to dynamically modulate its interaction with cellular partners. GRK2 can be phosphorylated by different kinases to either enhance -PKA [177], PKC [178, 179] and Src [180]- or decrease -ERK [181]- membrane targeting and/or the catalytic activity of GRK2 thus opening the possibility of transmodulation by different signaling pathways.

GRK2 can also be S-nitrosylated by NO, which resulted in the inhibition of GRK2 activity on β-AR signaling and other downstream targets [182].

5.3.2.b) Control of GRK2 levels

Regarding the regulation of GRK2 expression, increased levels of GRK2 could be promoted by an increase in mRNA levels, thereby suggesting that transcriptional control mechanisms are taking place, or by an imbalance between transcriptional and posttranscriptional mechanisms (protein stability) that can lead to increased GRK2 protein expression.

In aortic smooth muscle cells, the agents that induce physiological vasoconstriction and hypertrophy markedly enhance GRK2 promoter activity, whereas pro-inflammatory cytokines promote the opposite effect, suggesting that the expression of GRK2 is strongly controlled at the transcriptional level by the interplay between various signal transduction pathways [183]. TGF-β also induces GRK2 expression in VSMCs [184]. However, whether these mechanisms apply to other cell types such as cardiomyocytes in the CVS awaits further investigation. Nevertheless, in the myocardium, long-term in vivo stimulation of β-ARs results in the impairment of cardiac β-AR signaling and increases the level of expression (mRNA and protein) and activity of GRK2 but not those of GRK5 [185].

Moreover, regulation of GRK2 stability may provide an important mechanism for modulation of its expression levels. Our group has reported that GRK2 is rapidly degraded by the proteasome pathway, and that GRK2 ubiquitination and turnover is enhanced by β_2-AR activation as a result of phosphorylation of GRK2 by c-Src and MAPK in a β-arrestin-dependent manner [186, 187]. Besides, Mdm2, an E3-ubiquitin ligase involved in the control of cell growth and apoptosis, that promotes survival and attenuates hypertrophy of cardiac myocytes [188], plays a key role in GRK2 degradation. Mdm2 and GRK2 association and subsequent proteolysis are facilitated by the β-arrestin scaffold function

upon β_2-adrenergic receptor stimulation. The Mdm2 action on GRK2 is dependent on the previous phosphorylation of GRK2 at Ser670 by MAPKs, while tyrosine phosphorylation was dispensable [189]. On the contrary, activation of the PI3K/Akt pathway by agonists such as IGF-1 alters Mdm2 phosphorylation and triggers its nuclear localization thus hampering Mdm2-mediated GRK2 degradation and leading to enhanced GRK2 stability and increased kinase levels in epithelial cells [190].

The presence of β-agonists has been reported to up-regulate GRK2 mRNA, whereas ischemia might promote GRK2 degradation by the proteasome in some experimental models [191-193]. However, there is limited knowledge of the mechanisms modulating GRK2 expression in cardiovascular cells in pathological settings such as during metabolic syndrome.

5.3.3. Non-Canonical role: GRK2 beyond GPCR desensitization

In addition to its canonical GPCR kinase activity, emerging data suggest that GRK2 may have other functions by building an extensive network of interactions with a variety of signal transduction partners, in a stimulus, cell type, or context-specific way. For example, GRK2 has been reported to functionally interact with p38 [194], MEK [195], Epac [196], IκBα [197], Cdk2, Pin1 [164], caveolin [198] and HDAC6 [199] in several tissues, although we will focus here on the GRK2 interactome in a cardiovascular context.

During conditions of oxidative stress, ERK is activated and phosphorylates GRK2 at Ser670, facilitates the binding to Hsp90, which results in the translocation of GRK2 to the mitochondria and the decrease in mitochondrial Ca^{2+} uptake capacity which could play a role in the suggested pro-death effects of GRK2 [152].

Additionally, GRK2 is a microtubule-associated kinase that directly phosphorylates tubulin following β-AR stimulation [200] therefore GRK2 levels can affect agonist-induced internalization by a mechanism involving microtubule stability [201] Furthermore, GRK2 phosphorylation of tubulin facilitates its polymerization into microtubules, and an increased ratio of microtubules to tubulin in the heart has been reported to be pro-hypertrophic [202].

GRK2 could also bind to and phosphorylate Smad2/3, which could have a significant impact on TGFβ-mediated fibrosis and remodeling post MI [203]. GRK2 expression levels are up-regulated upon TGFβ stimulation in hepatic cells, initiating a negative feedback loop which results in the phosphorylation of Smad and impaired activation and translocation of this protein to the nucleus. Moreover, it has been reported that the TGFβ-mediated increase in GRK2 levels antagonizes Ang II-induced VSMC proliferation and migration, thus, GRK2 potentially mediates the crosstalk between both signaling cascades [184].

GRK2 can also interact directly with Akt in liver endothelial cells, reducing eNOS phosphorylation and NO production thus contributing to portal hypertension [204]. On the other hand, it was very recently described that GRK2 coprecipitates with eNOS

in mouse hearts and that ischemia and oxidative stress increases this interaction [205]. Interestingly, GRK2 and eNOS negatively regulate one another: eNOS promotes S-nitrosylation of GRK2 and GRK2 inhibits eNOS activation by an as yet unknown mechanism [205].

Finally, our group and others have contributed to the knowledge of GRK2´s role as modulator of insulin signaling by its interaction with IRS1. This is analyzed in detail in the *article #2* of the compended articles of this thesis which is entitled "G protein-coupled receptor kinase 2 (GRK2): A novel modulator of insulin resistance."

The role of GRK2 in the modulation not only of GPCRs but also of insulin signaling highlights the importance of GRK2 as a transversal target able to prevent the development of not one but several of the most important of the CV risk factors which coexistence result in maladaptation and lead to overt CVD.

6. CHOOSING THE APPROPRIATE MODEL OF STUDY

The physiological scenario that regulates CVS is complex and difficult to mimic in its extension in a closed system as an in vitro model. In vivo models are the only ones that allow to study changes in molecular signaling and tissue structure in association with organ function, and to model the effect of either physiological inputs or of stresses relevant to human disease on the CVS. For this reason, the most suitable way to study the mechanisms of adaptation of the CVS to different pathological outputs is to use animal models.

It is a challenge to directly compare the findings reported for human patients with those derived from animal studies. For example, cardiac physiology such as heart rate is different in rodents and humans but, although the murine heart is far smaller than the human, the heart to body weight ratio is similar in both species, as is the relative thickness of the right and left ventricular walls [206]. On the other hand rodent models are less likely to develop atherosclerotic disease in coronary arteries and spontaneous ischemia, which exists in many human patients with T2DM. Important differences also exist in the hormonal milieu and the concentration of lipids [31]. Regarding the vasculature, humans and mice share many features from the anatomical and histological point of view. However, they have important differences including the location of coronary arteries and the thickness of the walls of mouse arteries, much thinner compared to their human counterparts [206].

Nevertheless, animal models provide the opportunity to conduct mechanistic studies and allow for "proof of concept" experiments that are often impossible to perform in humans. Besides, end-organ effects of obesity that require a long-term treatment to become evident are much easier to study and observe in small rodents with a life span of 1-2 years that in other animal models more related to humans with a much longer life span.

Another important question to address is the experimental tools selected to modulate the levels or activity of the protein of study. Essentially there are two options: the use of a chronic or acute pharmacological stimulator or inhibitor, or, alternatively, utilize a genetically-engineered animal model.

Regarding pharmacological inhibitors of GRK2, much research effort has been dedicated to identify a selective compound that could inhibit GRK2 with enough potency and efficacy. Although there are several recent promising results, the drugs obtained so far are not selective for GRK2 [207-210]; have poor potency [211]; or have non drug-like properties like polyanions [212]. Takeda Pharmaceuticals, Inc. have developed potent inhibitors apparently selective for the GRK2/3 subfamily that bind the active site of the enzyme [213] but these have not advanced to pre- or clinical trials. Some authors use a commercial chemical compound (methyl[(5-nitro-2-furyl)vinyl]-2-furoate) in their studies but it its selectivity and efficacy towards the GRK2 isoform has not been tested solidly in vivo and thus off-target effects over other GRK isoforms or kinases cannot be discarded [214-220].

Taken all this into account, in this thesis we have used GRK2 hemizygous mice to study the effect that an overall decrease of GRK2 could have in the CVS pathophysiology. We chose to utilize male and middle-aged adult mice (9-10 months old) in order to minimize hormonal influence and to imitate the conditions in which CV events are first detected in the human setting, respectively (see below). To mimic primary hypertension we have used Ang II infusion during 7 days by osmotic minipumps and 12 weeks or 33 weeks of HFD feeding in order to promote diet-induced insulin resistance or obesity, respectively. Noteworthy, to test acute insulin sensitivity in the heart we have performed intravenous injections of insulin through the tail vein because this delivery route directly reaches the heart and more precisely mimics the effects of an internal burst in insulin levels produced by pancreatic cells although it is technically more difficult than other techniques such as intraperitoneal injection that are also more widely used. This administration route is also the most appropriate to detect the differences in insulin signaling in the very fast and acute phase of insulin signaling (first five minutes after injection).

6.1. GRK2+/- mice

The most suitable way to study the effects of a putative selective inhibitor of GRK2 at this moment is to use genetically-modified animal models. In this particular case we needed an animal model that could mimic the effects of a putative pharmacological GRK2 inhibitor that would systemically down-modulate the action of GRK2, but avoiding its complete abrogation, therefore we used the hemizygous GRK2+/- mice. We hypothesized that decreasing GRK2 levels would be beneficial to avoid the development of pathological CVS remodeling and dysfunction as has been shown to occur using transgenic βARKct mice and tissue-specific GRK2 knock outs under other pathological situations.

In the GRK2 hemizygous mice, in contrast to what happens in βARKct mice, we are studying not only the activity of GRK2 as a kinase but also its role as a scaffolding

protein. Also, as opposed to tissue-specific GRK2 knockout mice, in GRK2+/- animals the consequences of a GRK2 downregulation in other organs and tissues that directly affect CVD can be taken into account, so we consider it is nowadays the most suitable model to evaluate the importance of GRK2 as a therapeutic target.

6.2. Adult 9/10 month-old mice

The age of the animals is also another crucial step in the process of designing the best experimental system, particularly to try to more precisely mimic the onset of the MetS and of CVD in humans. Although the incidence of these pathological events is significantly higher in the adult-elderly population relative to young individuals, most of the preclinical studies are usually performed in very young animals (2-3 months old) that do not reproduce the physiological environment and conditions present in older animals, much less in adult or elderly human patients. We have used, even when more cost- and effort-demanding, 9 month-old mice in order to study the effects of decreasing GRK2 levels when CV events are first detected in humans, that is in adulthood. In fact, 9 month-old C57BL/6 wild-type mice exhibit a prediabetic state with significantly higher levels of blood glucose and insulin at baseline compared with 3 month-old mice [170], what more precisely resembles the clinical setting of age-derived MetS in human patients.

6.3. Ang II infusion-induced hypertension

To study the role of GRK2 in hypertension we have use an Angiotensin II chronic infusion model by means of mini osmotic pump intradermal implantation for seven days. Overactivation of RAS contributes to human hypertension in a majority of patients, therefore, the use of Ang II infusion to induce the experimental model is a well established approach resembling the human clinical setting. Apart from chronic Ang II infusion, there are other ways to induced RAS overactivation but they include invasive surgery (renovascular and neurogenic hypertension models), or take too much time to develop (dietary-induced hypertension) [221]. While this model is probably the most widely used pharmacological murine model for human hypertension, it remains different from the human setting since RAS overactivation frequently coexist in human patients with other environmental and genetic factors that help develop the hypertensive condition.

6.4. HFD-induced insulin resistance and obesity

To study the effect of GRK2 down-modulation in obese individuals we have chosen to use a diet-induced obesity model in which the animals are fed a high fat diet (30% of crude fat by weight which provides 51-54% of the total calories). This experimental design tries to mimic the human lifestyle in developed and in even some underdeveloped countries of the 21st century, where the obesity is mainly caused by an excess intake of calories from fat, whilst genetic models deficient in the leptin receptor (db/db) or leptin synthesis (ob/ob) are not fully representative of the varied and complex human pathophysiology of obesity. However, *stricto sensu*, diet-induced obesity cannot be strictly categorized as an animal model of metabolic syndrome because dieting an animal with high fat diet rarely causes the complete clinical picture that comprises the cardiovascular and

metabolic disease in humans. For example, in some cases, obesity-induced hypertension is achieved [222, 223], but this is not commonplace, and most research papers do not report an increase in blood pressure values after HFD feeding. There are several factors that could be implicated in these differences between different experimental approaches as the mouse genetic background, the diet and its percentage of fat, the gender, exercise vs sedentarism, and so on.

In sum, we have evaluated the efficiency of GRK2 as a therapeutic target against the development of hypertension and IR since this kinase has been identified as a key modulator of GPCR-dependent or independent pathways such as Ang II or insulin signaling pathways, related to the different characteristics of the CVD. The experimental work presented in this thesis unveils GRK2 as an integrative sensor that increases its levels upon several pathological inputs thus helping fuel metabolic and cellular vicious cycles with deleterious effects on the CVDC. Therefore, targeting GRK2 levels or activity could represent a feasible therapeutic strategy for the treatment or prevention of hypertension and insulin resistance under different conditions, able to stop the inexorable progression along the CVDC.

OBJECTIVES

GRK2 is emerging as a signaling hub which displays a very complex interactome and key roles in the regulation of both GPCR-dependent responses, such as those triggered by catecholamines or angiotensin, or in the insulin-modulated intracellular cascade. Moreover, increased GRK2 levels or functionality appear to be highly related to the onset or development of several cardiovascular diseases (CVD) and/or its comorbidities. Conversely, several studies have suggested a cardioprotective role of genetic GRK2 downmodulation or inhibition. However, the molecular mechanisms underlying both the deleterious and beneficial effects of such changes in GRK2 levels in cardiovascular function are not fully understood.

In order to shed new light on such mechanisms, the general objective of this work is to investigate the impact of altering GRK2 levels on key signaling pathways and phenotypic features under relevant CVD pathological contexts such as hypertension, age and high fat diet-induced obesity and insulin resistance. For this purpose, we have used as an experimental setting adult hemizygous GRK2 (GRK2+/-) mice, an animal model that would better mimic the conditions in which CV events are first detected in humans.

Specific objectives:

1. Analyze the impact of altered GRK2 genetic dosage on vascular responses, vascular structure and on the induction of a primary hypertension phenotype triggered by Angiotensin-II infusion in adult mice, and the molecular mechanisms involved.

2. Explore the interconnections among GRK2 dosage, cardiac insulin sensitivity, cardiac gene expression patterns and heart physiopathology in adult mice or animals fed with high-fat diet, two conditions known to promote insulin resistance.

3. Analyze the impact of altered GRK2 genetic dosage on the cardiac remodeling associated to obesity promoted by long-term high fat diet feeding in adult animals.

ARTICLES

INTRODUCTION TO ARTICLE #1

The first article presented is entitled Increased Nitric Oxide Bioavailability in Adult GRK2 Hemizygous Mice Protects Against Angiotensin II -Induced Hypertension (Hypertension. 2014; 63(2):369-75).

In order to investigate the impact of altering GRK2 levels on key signaling pathways related to vascular function and on the phenotypic features characteristic of hypertension, we set a collaborative project between our group and that of Prof. Mercedes Salaíces (Pharmacology Department, Faculty of Medicine, UAM), a renowned expert in the field. This was the first study aimed to characterize the effect of a systemic reduction in GRK2 levels on vascular structure and biomechanics, both in basal and in AngII-treated adult mice.

The responses of conductance and resistance arteries to different stimuli were investigated in adult 9 month-old mice of two different genotypes (WT and GRK2+/-) that differ in the systemic dosage of GRK2. The initial results pointing to an increased bioavailability of NO in GRK2 hemizygous mice, led us to analyze the phenotypic and biochemical features of these animals upon induction of primary hypertension by chronic Ang II infusion through miniosmotic pumps. We observed that lower levels of GRK2 are related to a preserved activation of the eNOS/Akt axis after a Ang II challenge, thus favoring an increased NO bioavailability, in turn leading to protection against the vascular remodeling and severe hypertension developed in wild-type mice.

My main contribution to this article (in which I am shared first co-author) was centered in the detailed molecular characterization of the signaling pathways and protein and RNA expression markers in the aortic vessels both in basal and Ang II-infused conditions. I also participated in the surgical implantation of the osmotic pump and in the follow-up of blood pressure evolution and heart rate measurements.

Increased Nitric Oxide Bioavailability in Adult GRK2 Hemizygous Mice Protects Against Angiotensin II–Induced Hypertension

María S. Avendaño,* Elisa Lucas,* María Jurado-Pueyo, Sonia Martínez-Revelles, Rocío Vila-Bedmar, Federico Mayor Jr, Mercedes Salaices, Ana M. Briones, Cristina Murga

Abstract—G protein–coupled receptor kinase 2 (GRK2) is a ubiquitous serine/threonine protein kinase able to phosphorylate and desensitize the active form of several G protein–coupled receptors. Given the lack of selective inhibitors for GRK2, we investigated the effects elicited by GRK2 inhibition in vascular responses using global adult hemizygous mice ($GRK2^{+/-}$). The vasodilator responses to acetylcholine or isoproterenol were increased in aortas and mesenteric resistance arteries from $GRK2^{+/-}$ mice compared with wild-type (WT) littermates. After angiotensin II (AngII) infusion, $GRK2^{+/-}$ mice were partially protected against hypertension, vascular remodeling, and mechanical alterations, even when resting basal blood pressures were not significantly different. AngII infusion also (1) increased GRK2 levels in WT but not in $GRK2^{+/-}$ vessels; (2) increased vasoconstrictor responses to phenylephrine in WT but not in $GRK2^{+/-}$ mice; and (3) decreased vasodilator responses to acetylcholine and vascular pAkt and eNOS levels more in WT than in $GRK2^{+/-}$ animals. Vascular NO production and the modulation of vasoconstrictor responses by endothelial-derived NO remained enhanced in $GRK2^{+/-}$ mice infused with AngII. Thus, $GRK2^{+/-}$ mice are resistant to the development of vascular remodeling and mechanical alterations, endothelial dysfunction, increased vasoconstrictor responses, and hypertension induced by AngII at least partially through the preservation of NO bioavailability. In conclusion, our results describe an important role for GRK2 in systemic hypertension and further establish that an inhibition of GRK2 could be a beneficial treatment for this condition. **(*Hypertension*. 2014;63:369-375.)** ● **Online Data Supplement**

Key Words: arteries ■ GRK2 ■ hypertension ■ nitric oxide

Different receptors and signaling molecules are involved in the development of hypertension by hyper-contracting or hypo-dilating blood vessels in a deleterious manner and by affecting the structure and mechanical properties of vessels. Among them, the G protein–coupled receptor (GPCR) family is of outmost importance. Adrenergic receptors and other GPCRs, such as angiotensin II (AngII), endothelin-1 (ET-1), dopamine, and vasopressin receptors, are key for vascular physiopathology.[1] AngII is a master regulator of vascular tone, and many animal models of hypertension are based on the chronic elevation of AngII levels.

GPCRs become inactivated to different extents when agonist signals are persistent in time, a process termed desensitization. This process is regulated by G protein–coupled receptor kinases (GRKs), a family of serine/threonine kinases able to phosphorylate intracellular domains of the receptors and initiate their uncoupling from the G protein, and thus signal termination.[2] Among the 7 GRK isoforms, GRK2 is the most abundant in vessels together with GRK5 and plays a determinant role in the control of systemic vascular responses.[3,4] The levels and activity of the GRK2 isoform are increased in animal models of hypertension and in lymphocytes from young patients with hypertension.[5] In addition, GRK2 mRNA levels, but not those of GRK3 or GRK5, increase in correlation with systolic blood pressure in humans.[6] GRK2 downregulates the in vivo effects of key vasoconstrictor receptors, such as ET[7] and AngII[8] receptors and $\alpha 1_D$ adrenoceptors.[9] Therefore, the increase in vascular GRK2 could have represented a protective mechanism for adaptation against a hypertensive phenotype. However, transgenic mice overexpressing GRK2 in the vascular smooth muscle cells (VSMC) show increased resting blood pressure.[8] Moreover, elevated GRK2 levels impair vasodilator β adrenoceptors responses in different tissues and animal

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From the Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid (UAM) (M.S.A., S.M.-R., M.S., A.M.B.), and Departamento de Biología Molecular and Centro de Biología Molecular "Severo Ochoa", UAM-CSIC (E.L., M.J.-P., R.V.-B., F.M., C.M.), Madrid, Spain; Instituto de Investigación Sanitaria La Paz, Madrid, Spain (M.S.A., S.M.-R., M.S., A.M.B.); and Instituto de Investigación Sanitaria La Princesa, Madrid, Spain (E.L., M.J.-P., R.V.-B., F.M., C.M.).

*These authors contributed equally to this work.

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Correspondence to Cristina Murga, Departamento de Biología Molecular, Centro de Biología Molecular "Severo Ochoa", Facultad de Ciencias, Universidad Autónoma de Madrid, Nicolás Cabrera 1, 28049 Madrid, Spain. E-mail cristina.murga@uam.es; or Ana M. Briones, Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo, 28029 Madrid, Spain. E-mail ana.briones@uam.es

models and, interestingly, in human patients treated with β-blockers GRK2 levels return to baseline.[4,10]

Classically, the preferential desensitization by GRK2 of vasodilator receptor subtypes versus vasoconstrictor ones has been invoked to explain the effect of upregulated GRK2 levels in human and in murine hypertension.[8] However, novel results implicating GRK2 in non–GPCR-dependent pathways prompt to redefine the role of GRK2 in the regulation of vascular tone. One such example is the described interaction of GRK2 with Akt that inhibits Akt-dependent activation of NO synthase, thus impairing NO production.[11] Another important emerging question is the relative importance of endothelial GRK2 as compared with VSMC-GRK2 because depletion of GRK2 in VSMC is unable to prevent portal hypertension.[9] Our work tries to shed some light on these issues describing for the first time that a global reduction in GRK2 causes resistance to the development of systemic hypertension in adult mice. This antihypertensive effect of GRK2 downregulation prevails even when responses to both vasodilator and vasoconstrictor receptors are enhanced and this is because of the increased NO bioavailability detected in GRK2$^{+/-}$ mice. Although extensive research has been performed on the effects of GRK2 overexpression or deletion in cardiac phenotypes, few studies have addressed how systemic changes in GRK2 levels can affect vascular function and to our knowledge, this is the first report to address the effect of GRK2 in vascular structure and biomechanics.

Methods

The Materials and Methods are described in the online-only Data Supplement.

Results

GRK2 Deficiency Increases Vasoconstrictor Responses Without Influencing Receptor Levels

Partial deficiency of GRK2 increased vasoconstrictor responses to phenylephrine in aortas from male (Figure 1A; Table S1 in the online-only Data Supplement) and female (Figure S1A; Table S1) mice. ET-1 (0.1 μmol/L)–mediated vasoconstrictor responses were also increased in aorta from male GRK2$^{+/-}$ mice compared with WT littermates (Figure S2A; Table S1). After repeated exposure to ET-1, vasoconstrictor responses were reduced in WT and GRK2$^{+/-}$ mice (Figure S2A). However, contractile responses to the second and third ET-1 administration remained larger in GRK2$^{+/-}$ compared with WT mice (Figure S2A), suggesting that partial deficiency in GRK2 prevents, in part, ET-1–induced desensitization. GRK2 deficiency did not modify AngII (1 μmol/L)–induced vasoconstrictor responses (Figure S2B; Table S1) or changed the reduced vascular responses after repeated exposure to AngII (Figure S2B).

GRK2$^{+/-}$ mice displayed decreased vascular GRK2 gene and protein expression (Figures S2C and S3), but no differences in protein or gene expression of AT1, AT2, ETA, ETB, or $\alpha 1_D$ receptors were observed between WT and GRK2$^{+/-}$ mice in aortas, and a reduction of only AT2 receptor protein was observed in mesenteric resistance arteries (MRA) from GRK2$^{+/-}$ mice (Figures S2C and S3).

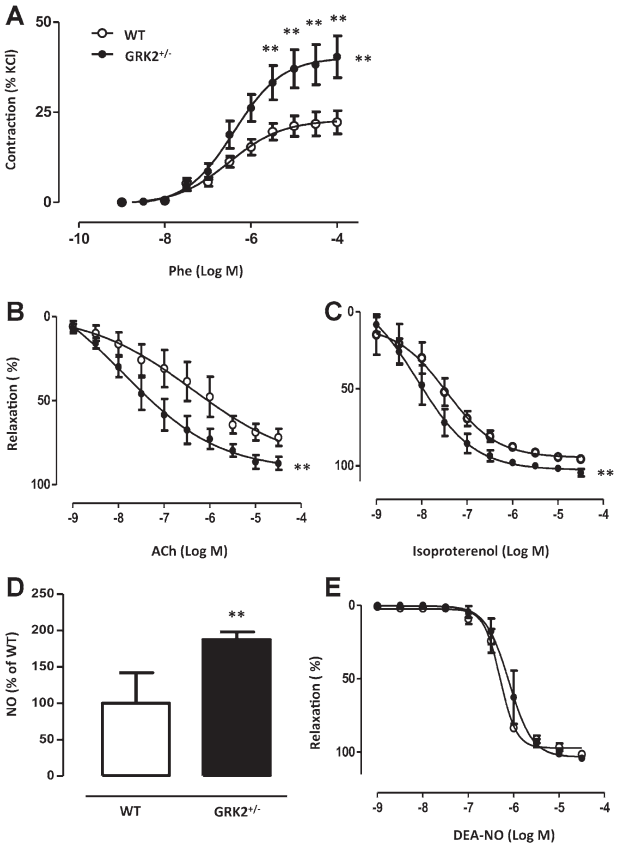

Figure 1. Effects of partial G protein–coupled receptor kinase 2 (GRK2) deficiency on vasoconstrictor and vasodilator responses and in NO production in aorta from male adult mice. Concentration–response curve to phenylephrine (Phe; **A**), acetylcholine (ACh; **B**), isoproterenol (**C**), and diethylamine NONOate (DEA-NO; **E**) and quantification of ACh-induced NO release (**D**) in aorta from male wild-type (WT) and GRK2$^{+/-}$ mice. n=5 to 10. $^{**}P<0.01$.

GRK2 Deficiency in Adult Mice Increases Endothelium-Dependent Vasodilator Responses and NO Release

The endothelium-dependent vasodilator responses induced by acetylcholine (ACh) or isoproterenol were increased in aorta from male and female GRK2$^{+/-}$ mice compared with WT mice (Figures 1B and 1C; Figure S1B and S1C). Accordingly, ACh-induced NO production was increased in aortas from GRK2$^{+/-}$ animals (Figures 1D; Figure S1D). In contrast, the endothelium-independent vasodilator responses induced by the NO donor diethylamine (DEA)-NO were similar in GRK2$^{+/-}$ and WT mice (Figures 1E; Figure S1E), suggesting that the observed differences are probably due to altered endothelium-mediated NO production. Similar results were observed in MRA from male and female mice (Figure S4A–S4F). ACh-induced NO production was also larger in MRA from male GRK2$^{+/-}$ mice (Figure S4G).

GRK2 Deficiency Reduces the Development of Hypertension and Prevents Vascular Remodeling After AngII Infusion

AngII infusion increased GRK2 levels in WT but not in GRK2$^{+/-}$ aortas (Figure 2A). Basal systolic blood pressure

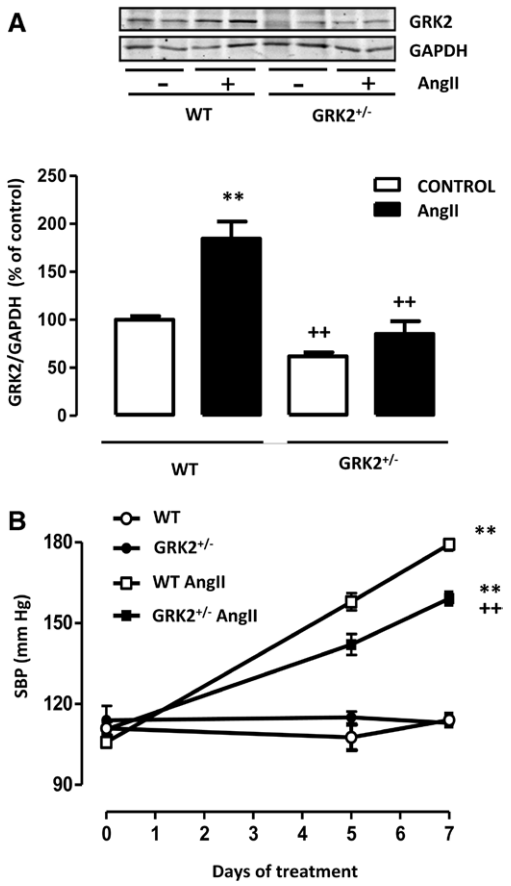

Figure 2. Partial G protein–coupled receptor kinase 2 (GRK2) deficiency reduces angiotensin II (AngII)–induced GRK2 expression and hypertension. **A**, Densitometric analysis and representative blot of GRK2 protein expression in aortic segments from wild-type (WT) and GRK2$^{+/-}$ mice treated or not with AngII. Data were normalized to the values of control WT littermates. **B**, Effect of AngII infusion on systolic blood pressure (SBP) in WT and GRK2$^{+/-}$ mice. Data represent mean±SEM. n=5 to 9. **P<0.01 vs untreated animals. ++P<0.01 vs WT either untreated (CONTROL) or treated with AngII.

(Figure 2B) and heart rate (Figure S5) were similar in untreated WT and GRK2$^{+/-}$ mice. Systemic infusion of AngII increased systolic blood pressure more in WT than in GRK2$^{+/-}$ mice (Figure 2B), but heart rate increase was indistinguishable between both groups (Figure S5).

Lumen and vessel diameter, wall thickness, and wall/lumen ratio of MRA were similar in untreated WT and GRK2$^{+/-}$ mice (Figure S6A–S6D), and vascular stiffness was also similar, as shown by the stress–strain relationship (Figure 3C), β parameter (WT, 4.87±0.3; GRK2$^{+/-}$, 4.98±0.37; n=5–6; P>0.05), and incremental distensibility (Figure S6E). After AngII infusion, lumen and vessel diameter were smaller in WT and similar or slightly larger, respectively, in GRK2$^{+/-}$ mice (Figure S7A–S7D). Wall thickness increased in both groups after AngII infusion (Figure S7E and S7F), and wall/lumen ratio was larger after AngII infusion in WT mice (Figure 3A) but not in GRK2$^{+/-}$ mice (Figure 3B). As expected, AngII increased vessel stiffness (Figure 3C, β parameter: WT, 8.31±0.51; GRK2$^{+/-}$, 6.34±0.35; n=5–7; P<0.05 versus control mice) and decreased distensibility (Figure S8) in MRA from both strains. However, these effects were less pronounced in GRK2$^{+/-}$ than in WT mice (Figure 3C; Figure S8; β parameter, P<0.05 GRK2$^{+/-}$ versus WT mice).

In aorta, lumen and vessel diameters were similar in WT and GRK2$^{+/-}$ mice irrespective of AngII infusion (data not shown). However, media thickness (Figure 3D) and media/lumen ratio (Figure 3E) increased after AngII only in WT and not in GRK2$^{+/-}$ mice, suggesting that partial deletion of GRK2 protects against AngII-induced vascular remodeling.

GRK2 Deficiency Improves Vascular Function and NO Signaling After AngII Infusion

AngII treatment increased vasoconstrictor responses to phenylephrine in WT mice, as described,[12] but not in GRK2$^{+/-}$ mice (Figure 4A and 4B). Because AngII infusion decreases NO availability in aorta,[12] the lack of effect of AngII on phenylephrine responses in GRK2$^{+/-}$ arteries might be related to a lesser decrease in NO. As shown in Figure 4C and 4D, the NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME; 100 µmol/L) enhanced phenylephrine contraction in GRK2$^{+/-}$ aortas more than in WT vessels after AngII (dAUC WT, 59±7; GRK2$^{+/-}$, 237±31; P<0.05), suggesting that NO bioavailability after AngII was better preserved in GRK2$^{+/-}$ aortas. Interestingly, endothelium removal also increased phenylephrine contraction more in aorta from AngII-infused GRK2$^{+/-}$ than WT mice (Figure S9; dAUC WT, 149±17; GRK2$^{+/-}$, 242±29; P<0.05), suggesting that in GRK2$^{+/-}$ mice there is an important contribution of the endothelium-derived vasodilator mediators, probably NO, on vascular contractile responses.

The endothelium-dependent vasodilator responses induced by ACh (Figure S10A and S10B) but not those triggered by the endothelium-independent vasodilator DEA-NO (Figure S10C and S10D) were reduced after AngII infusion. However, this deleterious effect of AngII infusion on endothelial function was less pronounced in GRK2$^{+/-}$ mice (% inhibition of maximal response induced by AngII; WT, 41.8±10; GRK2$^{+/-}$, 8.15±3; P<0.05). In agreement, aortic NO production induced by ACh (10 µmol/L) was greater in GRK2$^{+/-}$ than in WT mice infused with AngII (Figure 4E).

To determine whether the increase in NO bioavailability observed in aorta from GRK2$^{+/-}$ mice is a result of alterations of the eNOS-Akt pathway, we measured the activation of Akt (one of the most important upstream activators of eNOS) and eNOS levels. AngII infusion decreased Akt phosphorylation and eNOS protein expression in aortic homogenates from WT mice (Figure 4F and 4G). In contrast, we did not detect any statistically significant decrease in pAkt levels after AngII infusion in GRK2$^{+/-}$ mice and the reduction on eNOS expression was lower than that observed in WT mice (Figure 4F and 4G).

Discussion

Vascular responses of adult GRK2$^{+/-}$ mice were characterized as a surrogate model of the effects exerted by a long-awaited pharmacological GRK2 inhibitor. GRK2$^{+/-}$ mice show enhanced vasodilator and vasoconstrictor responses, with only particular differences toward certain contractile agonists. However, these mice are resistant to AngII-induced systemic hypertension, vascular remodeling, and

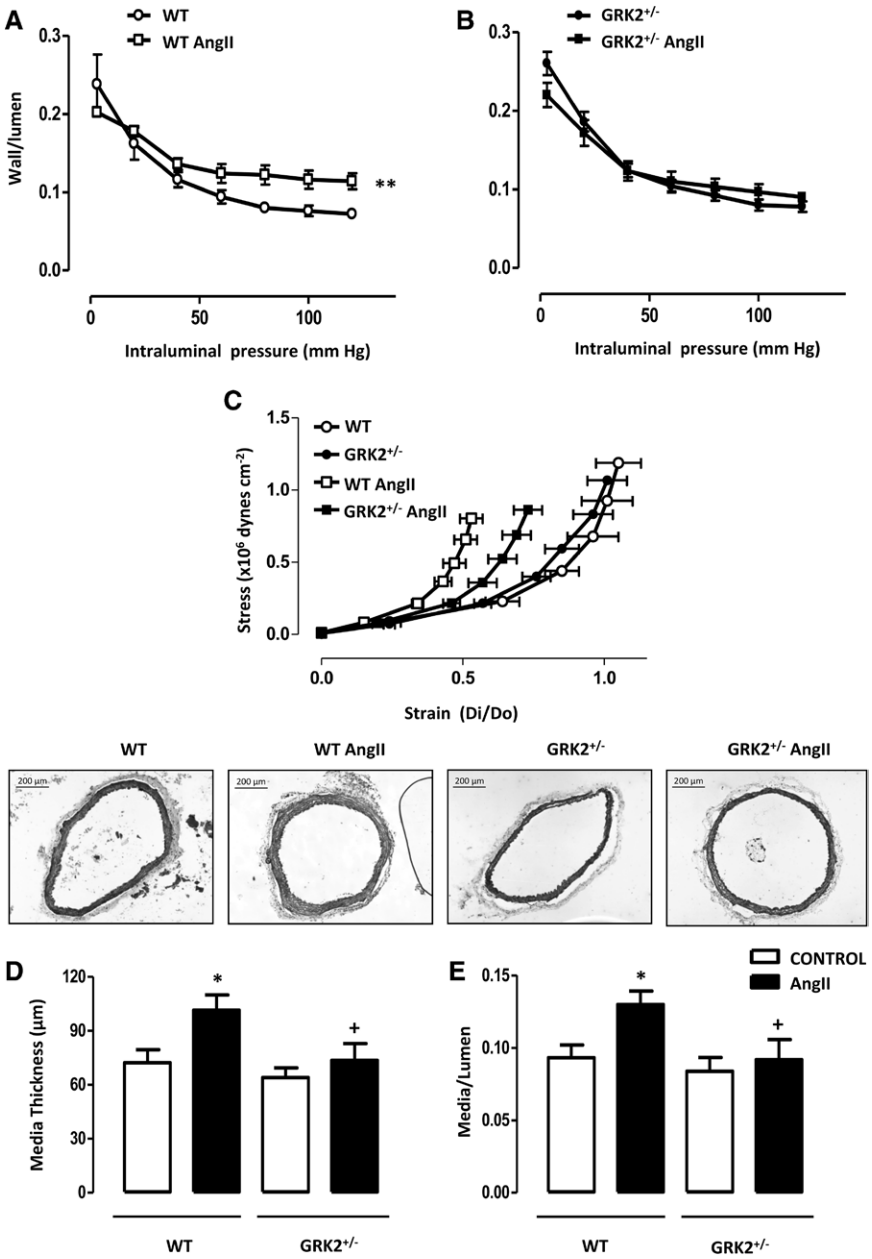

Figure 3. Partial G protein–coupled receptor kinase 2 (GRK2) deficiency protects against angiotensin II (AngII)–induced vascular remodeling and stiffness. Wall/lumen ratio (**A** and **B**) and stress–strain relationship (**C**) in mesenteric resistance arteries from untreated and AngII-infused wild-type (WT) and GRK2$^{+/-}$ mice. Media thickness (**D**) and media/lumen ratio (**E**) in aorta from WT and GRK2$^{+/-}$ mice untreated or treated with AngII. Representative photographs of hematoxylin-eosin aortic sections are shown. Data represent mean±SEM. n=5 to 11. *P<0.05, **P<0.01 vs untreated animals. +P<0.05 vs WT in the presence of AngII.

mechanical alterations. They better maintain endothelial function and display an attenuated AngII-induced decline in the Akt-eNOS route and in eNOS levels. These data further build on previous results describing a role for GRK2 in the control of portal hypertension[9,11] and in diabetes mellitus/obesity-triggered mild hypertensive conditions.[13] This report is the first to characterize the role of an overall selective inhibition of GRK2 function in the development of systemic hypertension and in the structure and biomechanics of the vessels.

Partial GRK2 deficiency is not enough to overcome the overt hypertensive phenotype completely achieved by chronic AngII infusion. However, results in diabetic and ob/ob mice[13] showed a full reversal of these types of mild hypertension by the use of a nonselective GRK2 inhibitor. This discrepancy might be explained by differences in the hypertensive models, the use of a wide spectrum GRK2 inhibitor as opposed to a reduction in of GRK2 levels in GRK2$^{+/-}$ mice, and by the lower blood pressure implicit to the diabetes mellitus/obesity study.[13] Also, our mice have been aged until an adult stage (9 months), a period more related to the human clinical setting of hypertension occurrence.

An apparent discrepancy exists between the fact that upregulation of GRK2 in mice models or hypertensive subjects causes elevated blood pressure and results establishing that increased GRK2 protein impairs vasoconstrictor signals.[8] In fact, when GRK2 levels are decreased, desensitization of certain GPCRs is impaired in vascular beds, but, in turn, GRK2 could be regulating vasodilator preferentially over vasoconstrictor receptors.[3] We observed that changes in vasoconstrictor responses induced by GRK2 deficiency depend on the agonist studied, whereas vasodilator ones are always increased in both sexes and in both conductance and resistance arteries. So, pathways alternative to

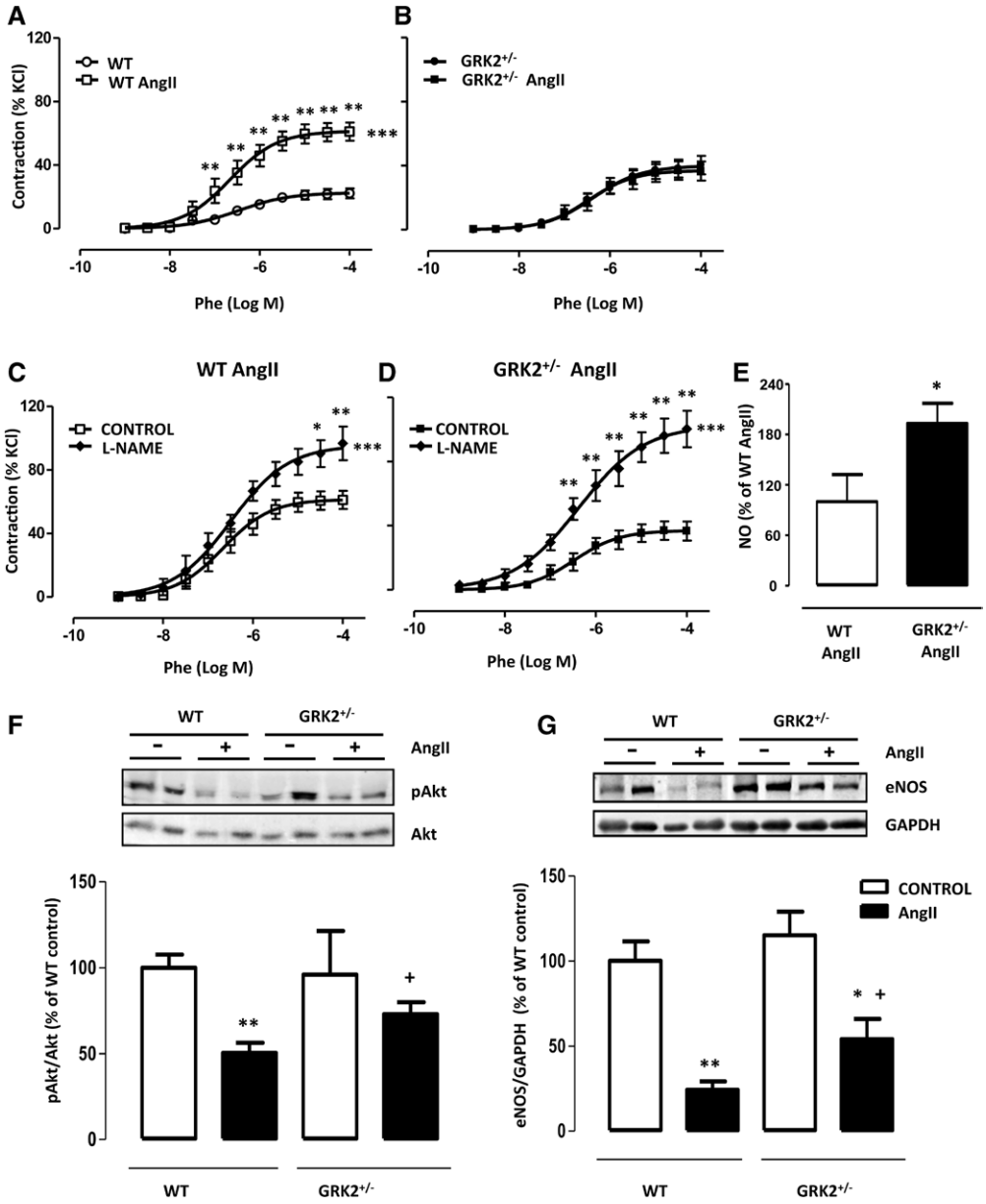

Figure 4. Partial G protein–coupled receptor kinase 2 (GRK2) deficiency protects against angiotensin II (AngII)–induced increased vasoconstriction by increasing endothelial-derived NO production. Effect of AngII infusion on the concentration–response curve to phenylephrine (Phe) in wild-type (WT) and GRK2$^{+/-}$ aortic segments (**A** and **B**). Effect of L-NAME (**C** and **D**) on the concentration–response curve to Phe in aortic segments from WT and GRK2$^{+/-}$ treated with AngII. **E**, Quantification of acetylcholine (ACh)-induced NO release in aortic segments from AngII-treated mice. Densitometric analysis and representative blots of phosphoAkt (pAKt) and Akt (**F**) and eNOS and GAPDH (**G**) in WT and GRK2$^{+/-}$ aortas from mice untreated or treated with AngII. Results are expressed as ratio of phosphoAkt to total Akt or between eNOS to GAPDH and normalized to values obtained for untreated WT mice. Data represent mean±SEM. n=4 to 13. **P*<0.05, ***P*<0.01, ****P*<0.001 vs the corresponding control. +*P*<0.05 vs WT infused with AngII.

GPCR-derived routes could be playing a role. For instance, GRK2 was described to interact with Akt, to impair eNOS activation, and to decrease NO bioavailability and GRK2 silencing reducing renal portal hypertension by increasing the Akt-NOS route,[11] and also a role for GRK2-mediated regulation of NOS in maintaining vascular responses,[14] and during diabetic- or obesity-triggered changes in blood pressure has been reported.[13] Our results represent the first demonstration that NO bioavailability is key to explain the antihypertensive phenotype derived from GRK2 down-regulation because NO production is increased basally in GRK2$^{+/-}$ aortas and mesenteric arteries, and adult GRK2$^{+/-}$ mice are capable of attenuating the AngII-induced drop in NO production much more efficiently than WT littermates. Accordingly, endothelium removal or L-NAME incubation enhanced phenylephrine contraction more in AngII-infused GRK2$^{+/-}$ than in WT aortas. Also, GRK2$^{+/-}$ mice are partially protected from AngII-induced vascular stiffness and remodeling, important determinants of high blood pressure,[15] both in the resistance and in the conductance vasculature. Of note, an increase in GRK2 protein levels in hypertensive WT but not GRK2$^{+/-}$ vessels is observed. This could be ascribed to

the lower hypertension detected in GRK2$^{+/-}$ animals and supports a pathological role of elevated vascular GRK2 levels in the hypertensive phenotype.

An increased basal blood pressure is found in mice overexpressing GRK2 in VSMC,[8] whereas mice deficient in muscular GRK2 show a lack of resistance to renal-induced hypertension.[9] Thus, the restoration of vasodilation achieved by VSMC-GRK2 targeting may not be sufficient to overcome the blunted relaxation responses elicited by endothelial cells. Moreover, Cohn et al[9] described no changes in vasoconstrictor AngII-induced vascular responses in endothelial-deprived vessels or in AngII-induced acute increases in blood pressure in VSMC-GRK2–deficient mice. In contrast, our results using global GRK2$^{+/-}$ mice demonstrate that these animals are partially resistant to AngII-induced hypertension. Comparison of these results clearly establishes that to achieve a therapeutic effect, endothelial GRK2 should be targeted, and that the control of NO bioavailability by GRK2 is crucial. GRK2 directly binds and inhibits Akt,[11] and we detect a maintenance of Akt activation after AngII treatment only in GRK2$^{+/-}$ mice, whereas WT animals efficiently inhibit this route. This preserved activation of Akt is also detected in other tissues in GRK2 hemizygous mice during aging-induced, high fat diet–induced, or tumor necrosis factor α–induced insulin resistance.[16] These changes cannot be tampered by changes in total Akt protein because, as opposed to other systems,[13] we do not detect significant changes in total Akt levels but rather an attenuation of the AngII-induced decline in total eNOS levels. Recently published studies have identified a novel role for GRK2 in mitochondria function and biogenesis. GRK2 removal from skeletal muscle cells reduces ATP production and impairs tolerance to ischemia.[17] However, elevated mitochondrial GRK2 in cardiomyocytes increases cellular injury, thus identifying GRK2 as a prodeath kinase.[18]

In conclusion, a partial deficiency of GRK2 differentially alters vasoconstrictor responses to different agonists. Nevertheless, vasodilator responses seem to be homogeneously increased in GRK2$^{+/-}$ arteries what probably explains the lack of changes in basal blood pressure observed in GRK2$^{+/-}$ mice. In addition, vascular structure and mechanics are similar in both genotypes. However, after an AngII challenge, lower GRK2 levels help to maintain the activation of eNOS through the Akt pathway, thus leading to preserved NO bioavailability. In this situation, NO-dependent vasodilation overcomes constriction which, together with the lesser deterioration of vascular structure and mechanics, explains why GRK2$^{+/-}$ mice are resistant to hypertension (Figure S11) and further highlights GRK2 as a therapeutic target for hypertension.

Perspectives

Our results provide novel evidences suggesting that a global GRK2 decrease could become an efficient treatment for hypertension and further highlight the importance of targeting endothelial GRK2 for an effective control of this condition, thus establishing that GRK2 targeting needs to include the endothelial compartment if reversal of hypertension is to be achieved.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Partial deficiency in G protein–coupled receptor kinase 2 (GRK2) affects vasoconstrictor responses and desensitization in a differential manner depending on the agonist studied without apparent changes in receptor levels. However, endothelium-dependent vasodilator responses are homogeneously increased in adult GRK2$^{+/-}$ conductance and resistance arteries via an increased NO availability.
- After AngII challenge, the lower GRK2 levels in adult GRK2$^{+/-}$ mice maintain the activation of eNOS by preserving phosphorylation of Akt. This better maintains NO bioavailability and protects vascular function in GRK2$^{+/-}$ mice. These facts, together with a less deteriorated vascular structure and mechanics after AngII challenge in GRK2$^{+/-}$ mice, might explain their resistance to the development of hypertension.
- This is the first report to characterize the effect of a systemic reduction in GRK2 levels on vascular structure and biomechanics, both in basal and in AngII-treated adult mice.

What Is Relevant?

- Our results provide new evidences for a novel therapeutic effect of lowering GRK2 levels/activity through the modulation of vascular function and NO bioavailability.

Summary

Partial deficiency of GRK2 differentially alters vasoconstrictor responses to different agonists, whereas vasodilator responses are homogeneously increased. After an AngII challenge, GRK2$^{+/-}$ mice maintain endothelial function, exhibit diminished vasoconstrictor responses, and display improved vascular structure and vessel stiffness compared with age-matched wild-type littermates. Moreover, GRK2$^{+/-}$ mice display an impaired AngII-induced decline in both the activation of the Akt-eNOS route and in total levels of the eNOS protein, thus leading to a preserved NO availability and a resistance to the development of hypertension.

ONLINE SUPPLEMENT

INCREASED NITRIC OXIDE BIOAVALIABILITY IN ADULT GRK2- HEMIZYGOUS MICE PROTECTS AGAINST ANGIOTENSIN II-INDUCED HYPERTENSION

María S. Avendaño*[,1,2], Elisa Lucas*[,3,4], María Jurado-Pueyo[3,4], Sonia Martínez-Revelles[1,2], Rocío Vila-Bedmar[3,4], Federico Mayor Jr.[3,4], Mercedes Salaices[1,2], Ana M. Briones[&,1,2], Cristina Murga[&,3,4]

*Equal contribution

[&]Corresponding authors

[1]Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, Spain

[2]Instituto de Investigación Sanitaria La Paz, Madrid, Spain.

[3]Departamento de Biología Molecular and Centro de Biología Molecular "Severo Ochoa", Universidad Autónoma de Madrid and CSIC, Spain

[4]Instituto de Investigación Sanitaria La Princesa, Madrid, Spain

Short title: GRK2 deficiency prevents hypertension development

Corresponding authors:

Dr. Cristina Murga
Dpto. Biología Molecular, Centro de Biología Molecular "Severo Ochoa"
Facultad de Ciencias. Universidad Autónoma de Madrid
Nicolás Cabrera 1, 28049-Madrid, Spain
Phone: 34911964641
Fax: 3491196 44 20
cristina.murga@uam.es

Dr. Ana M. Briones
Dpto. Farmacología
Facultad de Medicina. Universidad Autónoma de Madrid
Arzobispo Morcillo 2, 28029-Madrid, Spain
Phone: 34914975399
Fax: 3491497 53 80
ana.briones@uam.es

METHODS

Animal models

Male and female mice either wild type (WT) or GRK2 hemizygous ($GRK2^{+/-}$) were generated on the C57BL/6 genetic background as described.[1] Four groups of 9 month-old mice were used: 1) WT; 2) $GRK2^{+/-}$; 3) WT infused with AngII (1.44 mg/Kg/day, 1 week, subcutaneously by osmotic minipumps, Alza Corp., CA, USA); 4) $GRK2^{+/-}$ infused with AngII. In a pilot study, we observed that minipump surgery followed by infusion with saline did not alter systolic blood pressure or vascular contractile responses in WT or $GRK2^{+/-}$ mice (data not shown).

Blood pressure and heart rate were measured by tail-cuff plethysmography. The Animal Care and Use Committee of our Institution, according to the guidelines for ethical care of experimental animals of the European Community, approved all experimental procedures. The study was conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and the current Spanish and European laws (RD 223/88 MAPA and 609/86).

Reactivity Experiments

Reactivity of mouse aorta and first-order branches of the mesenteric artery (MRA) was studied in a wire myograph, as previously described.[2] After a 30-min equilibration period in oxygenated Krebs Henseleit solution (KHS), arterial segments were stretched to their optimal lumen diameter for active tension development. Contractility of segments was then tested by an initial exposure to KCl (120 mM). The presence of endothelium was determined by the ability of 10 µmol/L acetylcholine (ACh) to relax arteries precontracted with phenylephrine at approximately 50% K^+-KHS contraction. Concentration-response curves to ACh, isoproterenol or diethylamine NONOate (DEA-NO) were performed in segments precontracted with phenylephrine. In other segments, concentrations response curves to phenylephrine in the presence or the absence of L-N^G-Nitroarginine Methyl Ester (L-NAME, 100 µmol/L) were performed. L-NAME was added 30 min before phenylephrine. A single concentration-dependent curve was performed in each segment. In other set of experiments, three consecutive administration of Angiotensin II (1 µmol/L) or Endothelin-1 (0.1 µmol/L) were given, at intervals of 30 min, with removal always after each dose of the drug present in the medium. In a separate group of arteries, endothelial layer was mechanically removed by rubbing the intimal surface.

Vasoconstrictor responses were expressed as a percentage of the tone generated by KCl. KCl-induced responses were similar in aorta from WT and $GRK2^{+/-}$ male and female mice (Table S1). Vasodilator responses were expressed as a percentage of the previous tone generated by phenylephrine. To compare the effect of L-NAME or endothelial removal on phenylephrine responses in segments from the different experimental groups, some results were expressed as 'differences of area under the concentration-response curves' (dAUC) in the absence and the presence of L-NAME or in the presence and in the absence of endothelial layer. AUCs were calculated from the individual concentration-response curve plots; the differences were expressed as a percentage of the AUC of the corresponding control situation.

NO Release

After an equilibration period of 60 min in HEPES buffer (in mmol/L: NaCl 119; HEPES 20; $CaCl_2$ 1.2; KCl 4.6; $MgSO_4$ 1; KH_2PO_4 0.4; $NaHCO_3$ 5; glucose 5.5; Na_2HPO_4 0.15; pH 7.4) at 37°C, aortic and mesenteric arteries segments of all the experimental conditions were incubated with the fluorescent probe 4,5-diaminofluorescein (DAF-2; 2 µmol/L) for 45 min. Then, the medium was collected to measure basal NO release. Afterwards these segments were incubated with phenylephrine 1 µmol/L and relaxed with ACh 10 µ µmol/L. At the end of this stimulation, the medium was collected to measure induced NO release. The fluorescence of the medium was measured at room temperature using a spectrofluorimeter (FLUOstar OPTIMA BMG LABTECH) with excitation wavelength set at 492 nm and emission wavelength at 515 nm.

The induced NO release was calculated by subtracting basal NO release from that evoked by ACh. Also, blank samples were collected in the same way as from segment-free medium in order to correct for background emission. The amount of NO released was expressed as arbitrary units/mg tissue. Data were expressed as % of results obtained for WT mice.

Pressure myography

The structural and mechanical properties of MRA were studied with a pressure myograph (Danish Myo Tech, Model P100, J.P. Trading I/S, Aarhus, Denmark), as previously described.[3] Briefly, the vessel was placed on two glass microcannulae and secured with surgical nylon suture. After any small branches were tied off, vessel length was adjusted so that the vessel walls were parallel without stretch. Intraluminal pressure was then raised to 120 mm Hg and the artery was unbuckled by adjusting the cannulae. The segment was then set to a pressure of 45 mm Hg and allowed to equilibrate for 60 min at 37ºC in calcium-free Krebs Henseleit Solution ($0Ca^{2+}$ KHS; omitting calcium and adding 1 mmol/L EGTA) intra and extra-vascular perfused gassed with a mixture of 95% O_2 and 5% CO_2. Intraluminal pressure was reduced to 3 mm Hg. A pressure-diameter curve was obtained by increasing intraluminal pressure in 20 mm Hg steps between 3 and 120 mm Hg. Internal and external diameters were continuously measured under passive conditions (Di0Ca, De0Ca) for 3 min at each intraluminal pressure. The final value used was the mean of the measurements taken during the last 30 seconds when the measurements reached a steady state.

Calculation of passive structural and mechanical parameters

From internal and external diameter measurements in passive conditions the following structural and mechanical parameters were calculated:

Wall:lumen =(De0Ca-Di0Ca)/ 2Di0Ca

Circumferential wall strain (ε)= (Di0Ca -D00Ca)/D00Ca, where D00Ca is the internal diameter at 3 mm Hg and Di0Ca is the observed internal diameter for a given intravascular pressure both measured in $0Ca^{2+}$ medium.

Circumferential wall stress (σ)= (P x Di0Ca)/(2WT), where P is the intraluminar pressure (1 mm Hg = 1.334×10^3 dynes/cm^2) and WT is wall thickness at each intraluminar pressure in $0Ca^{2+}$-KHS.

Arterial stiffness independent of geometry is determined by the Young's elastic modulus (E=stress/strain). The stress-strain relationship is non-linear; therefore, it is more appropriate to

obtain a tangential or incremental elastic modulus (Einc) by determining the slope of the stress-strain curve (Einc=δσ/δε). Einc was obtained by fitting the stress-strain data from each animal to an exponential curve using the equation:

$\sigma = \sigma_{orig} e^{\beta\varepsilon}$ where σorig is the stress at the original diameter (diameter at 3 mmHg). Taking derivatives on the above equation we see that Einc=βσ. For a given σ value, Einc is directly proportional to β. An increase in β implies an increase in Einc which means an increase in stiffness.

Histological analysis and vessel morphometry

Aortas were fixed with 4% paraformaldehyde and embedded in Tissue Tek OCT medium. 10-μm cross sections from fixed aortas were stained with hematoxylin-eosin. All images were acquired at room temperature using a microscope (DM2000; Leica) with 10× objective. Morphometric determinations of the lumen and vessel areas were performed by using Metamorph image analysis software (Universal Imaging, Molecular Devices Corp. Downingtown, PA, USA). All microscopic images of the sections were traced for the calculations of the areas. To determine the luminal area, the cross-sectional area enclosed by the internal elastic lamina was corrected to a circle by applying the form factor $l^2/4\pi$ to the measurement of the internal elastic lamina, where l is the length of the lamina. Vessel area was determined by the cross-sectional area enclosed by the external elastic lamina corrected to a circle, applying the same form factor ($l^2/4\pi$) to the measurement of the external elastic lamina. The media area was calculated as the difference between the corrected vessel and luminal areas. Internal and external diameters were calculated from luminal and vessel areas, respectively. This method avoids miscalculations of areas caused by eventual collapse of the immersion-fixed arteries.[4]

Western Blot

Frozen aortas and mesenteric arteries were pulverized using liquid nitrogen and a smasher and homogenized in RIPA buffer (100 mmol/L Tris-HCl pH 7.4, 0.6 mol/L NaCl, 2% Triton x-100 (v/v), 0.2% SDS, 1% DOC) completed with protease and phosphatase inhibitors (100 μmol/L PMSF, 1 μmol/L Benzamidine, 10 μg/mL STI, 16 μU Aprotinine, 10 μg/mL Bacitracine and Phosphatase Inhibitor Cocktail (PhosSTOP, Roche) following the manufacturer´s protocol for 1 h. at 4ºC. Next, samples were centrifuged at maximum speed and supernatants were quantified. To determine protein expression, 30-40 μg of protein were resolved on a 7.5% SDS-PAGE gel and transferred to nitrocellulose. The membrane was immunoblotted using primary antibodies against eNOS (BD Transduction Laboratories) (1:1000), phospho-Akt (Ser473) (Cell Signaling) (1:1000), Akt (Cell Signaling) (1:1000), GRK2 (Santa Cruz) (1:1000), AT1 (Santa Cruz) (1:200), AT2 (Santa Cruz) (1:200) and GAPDH (Abcam) (1:5000). Blots were developed using fluorescently labelled secondary antibodies and measured the signal on a LiCOR Odyssey scanner. Data were normalized to GAPDH or total protein values, and expressed as % over results obtained for untreated or WT mice.

qRT-PCR

mRNA from frozen aortas were extracted using metal beads (2 min, 30 Hz) in a Tissue Lyser and Fibrous Tissue RNeasy Mini Kit, both from QIAGEN. For quantitative PCR, total RNA was reverse transcribed using SuperScript® III First-Strand Synthesis System (Invitrogen) according manufacturer´s protocol. cDNAs were quantified by real-time PCR on an Applied Biosystems 7900HT Fast Real-Time PCR System, using specific primers for mouse *Adra1d*: 5′-GTC TTC GTC CTG TGC TGG TT-3′ and 5′-CTT GAA GAC GCC CTC TGA TG-3′, *Agtr1a*: 5′-TCT GCT GCT CTC CCG GAC T-3′ and 5′-TGC TGT GAG TTA TCC CAG ACA AAA TG-3′, *Agtr1b*: 5′-GTG ACA TGA TCC CCT GAC AGT-3′ and 5′-AGT GAG TGA ACT GTC TAG CTA AAT GC-3′, *Agtr2*: 5′-GGG AGC TGA GTA AGC TGA TTT ATG A-3′ and 5′-AGC AAC TCC AAA TTC TTA CAC CTT TTT A-3′, *Ednr*a: 5′-ACC CTC GTT CTC CAG CTC A-3′ and 5′-TTG GTC TCA CGC CTT TCT TT-3′, *Ednrb*: 5′-AAT GGT CCC AAT ATC TTG ATC G-3′ and 5′-TCC AAA TGG CCA GTC CTC T-3′, *Adrbk1:* 5′-GCA GTT TGT CCT GCA GTG TG -3′ and 5′-TTC ATC TTG GGT ACT CGC TGT-3′, *Hprt*: 5′-CCT GGT TCA TCA TCG CTA ATC-3′ and 5′-TCC TCC TCA GAC CGC TTT T-3′, *Rps29*: 5′-CTG AAC ATG TGC CGC CAG T-3′ and 5′-TCA AGG TCG CTT AGT CCA ACT TAA T-3′, *Gapdh:* 5′-CTC CCA CTC TTC CAC CTT CG-3′ and 5′-CAT ACC AGG AAA TGA GCT TGA CAA-3′, *β-actin*: 5′-CTA AGG CCA ACC GTG AAA AG-3′ and 5′-ACC AGA GGC ATA CAG GGA CA-3′ and *18S*: 5′-CTC AAC ACG GGA AAC CTC AC-3′ and 5′-CGC TCC ACC AAC TAA GAA CG-3′. PCR amplification was performed in a volume of 10 µl containing 2.5 µmol/L of each primer and 5 µl Power Sybr Green PCR Master Mix (Applied Biosystems). The conditions were 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C, followed by 15 s at 95 ºC, 15 s at 60 ºC and 15 s at 95 ºC. *Adra1d, Agtr1a, Agtr1b, Agtr2, Ednra* and *Ednrb* mRNA levels were subsequently normalized to *Hprt, Rps29* and *Gapdh* mRNA which were the better for normalization according NormFinder software because of their stability through the different groups under study. The results obtained were analysed by GenEx 5.3.7 software.

Data Analysis and Statistics

All data are expressed as mean values±SEM and *n* represents the number of animals. Results were analyzed by two-way ANOVA for repeated measures followed by Bonferroni's *post hoc* test for the concentration response curves data, and by unpaired Student's *t*-test, Mann-Whitney or one-way ANOVA for the other measurements. Statistical symbols in the concentration response curves indicate differences in the two way ANOVA when placed close to the curve and differences in the *post hoc* test when placed above or below individual doses. A $p<0.05$ was considered significant.

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Table S1. Effect of partial GRK2 deletion ($GRK2^{+/-}$) on vasoconstrictor responses in aortic rings from male and female mice.

	Male		**Female**	
Vasoconstrictor drug	**WT**	**$GRK2^{+/-}$**	**WT**	**$GRK2^{+/-}$**
KCl (mN/mm)	2.50±0.31	2.82±0.45	2.59±0.12	2.61±0.19
Phe (Emax) (mN/mm)	0.69±0.12	1.14±0.17*	1.25±0.18	1.83±0.16*
ET-1 (Emax) (mN/mm)	0.08±0.05	0.26±0.05*	-	-
Ang II (Emax) (mN/mm)	0.07±0.02	0.08±0.06	-	-

Data are expressed as mean±SEM. n=4-12. *P<0.05 vs WT.

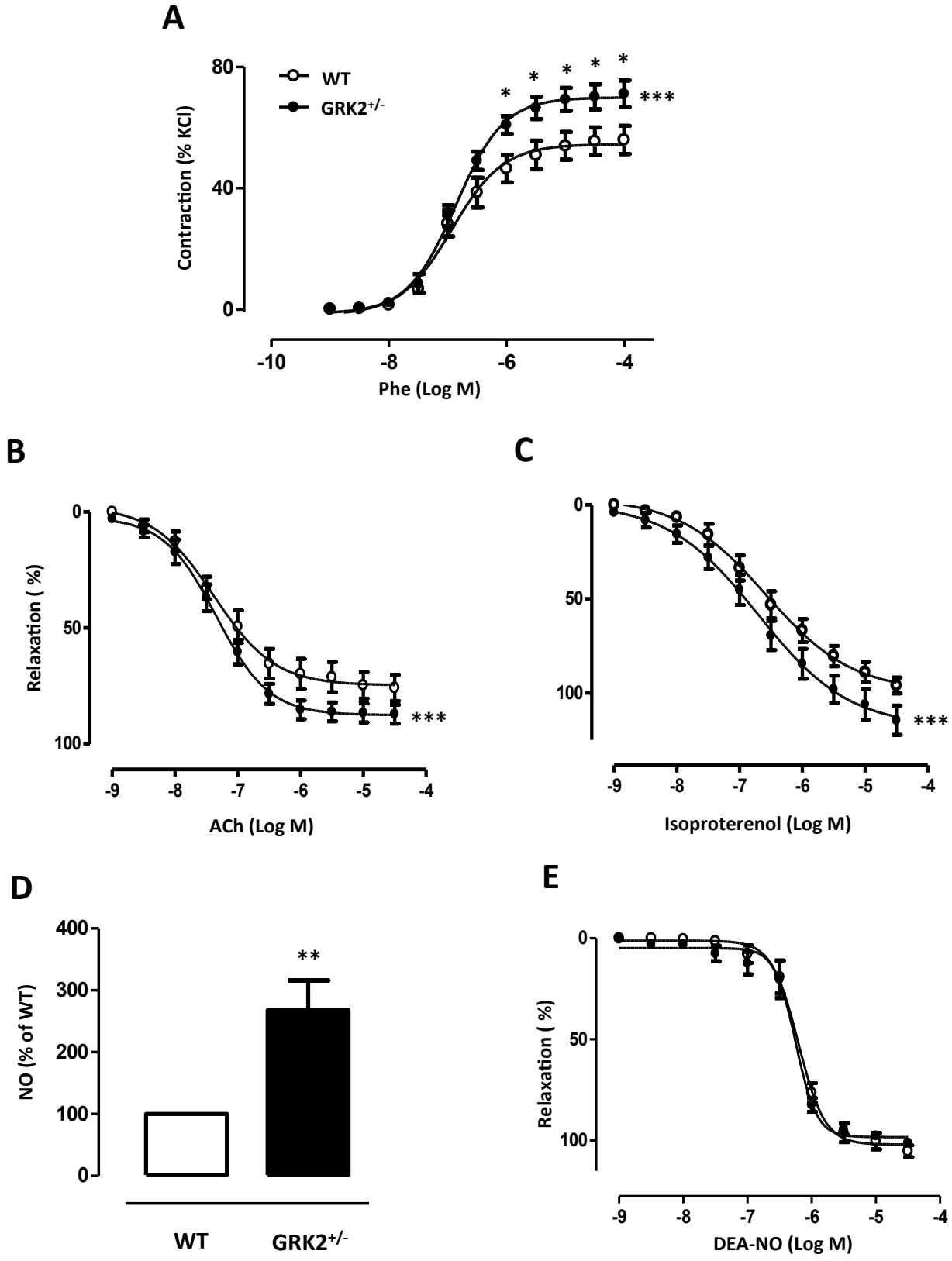

Figure S1. Effects of partial GRK2 deficiency on vasoconstrictor and vasodilator responses and in NO production in aorta from female adult mice. Concentration-response curve to phenylephrine (Phe) (A), acetylcholine (ACh) (B), isoproterenol (C), and diethylamine NONOate (DEA-NO) (E) and quantification of ACh-induced NO release (D) in aorta from female wild type (WT) and GRK2$^{+/-}$ mice. n=7-10. *P<0.05, **P<0.01, ***P<0.001.

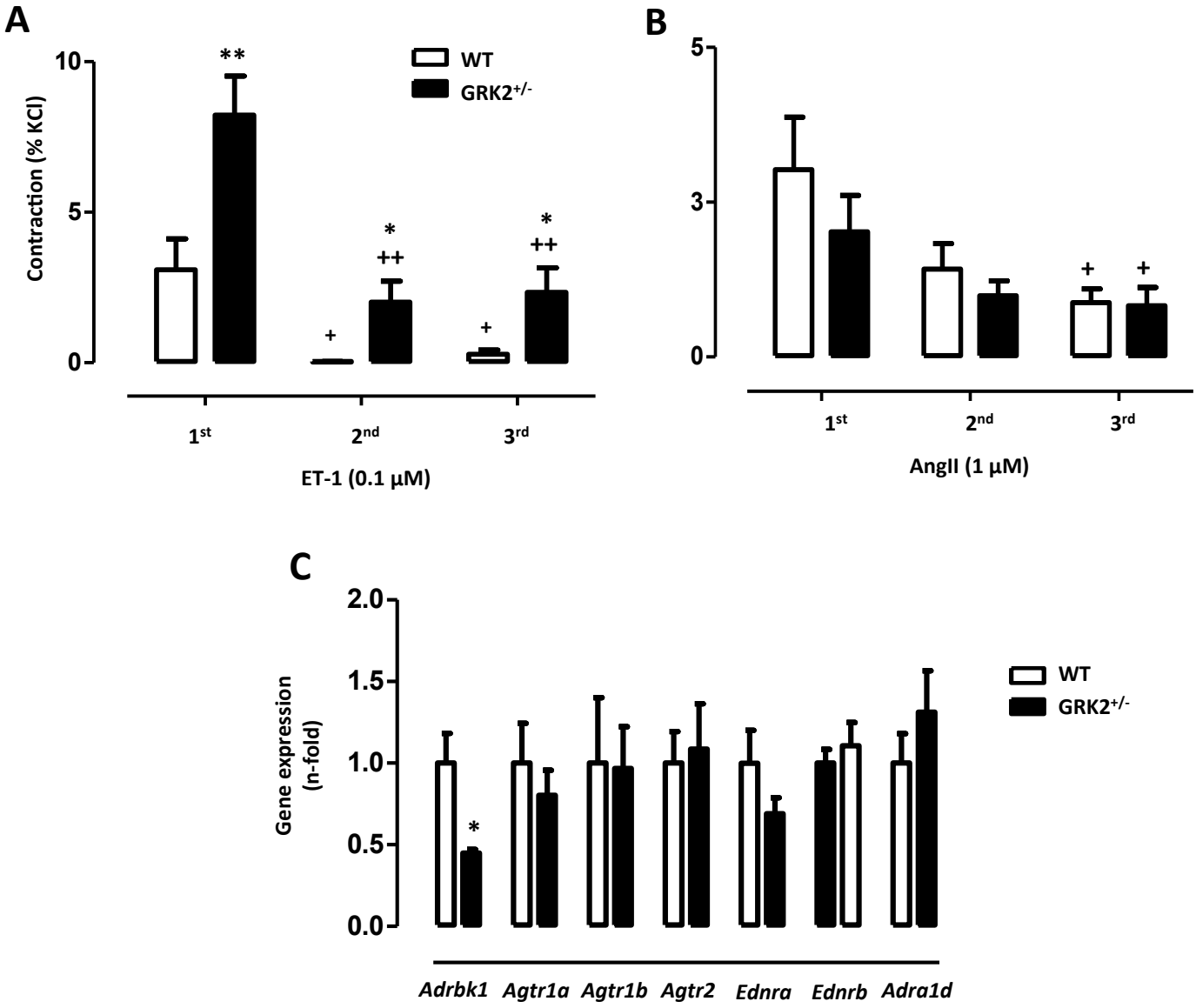

Figure S2. Vasoconstrictor responses to endothelin-1 (ET-1) (A) and Angiotensin II (AngII) (B) administered three consecutive times at intervals of 30 min in aortas from WT and GRK2 $^{+/-}$ mice. (C) Gene expression of GRK2 (*Adrbk1*), Angiotensin receptors type 1a and b and 2 (*Agtr1a, Agtr1b, Agtr2*), endothelin-1 A and B receptors (*Ednra, Ednrb*) and $\alpha 1_D$ receptor (*Adra1d*) in aorta from WT and GRK2$^{+/-}$ adult mice. Data were normalized to values of WT mice. Data represent mean±SEM. n=5-9. *P<0.05; **P<0.01 vs WT. +p<0.05, ++p<0.01 vs first administration.

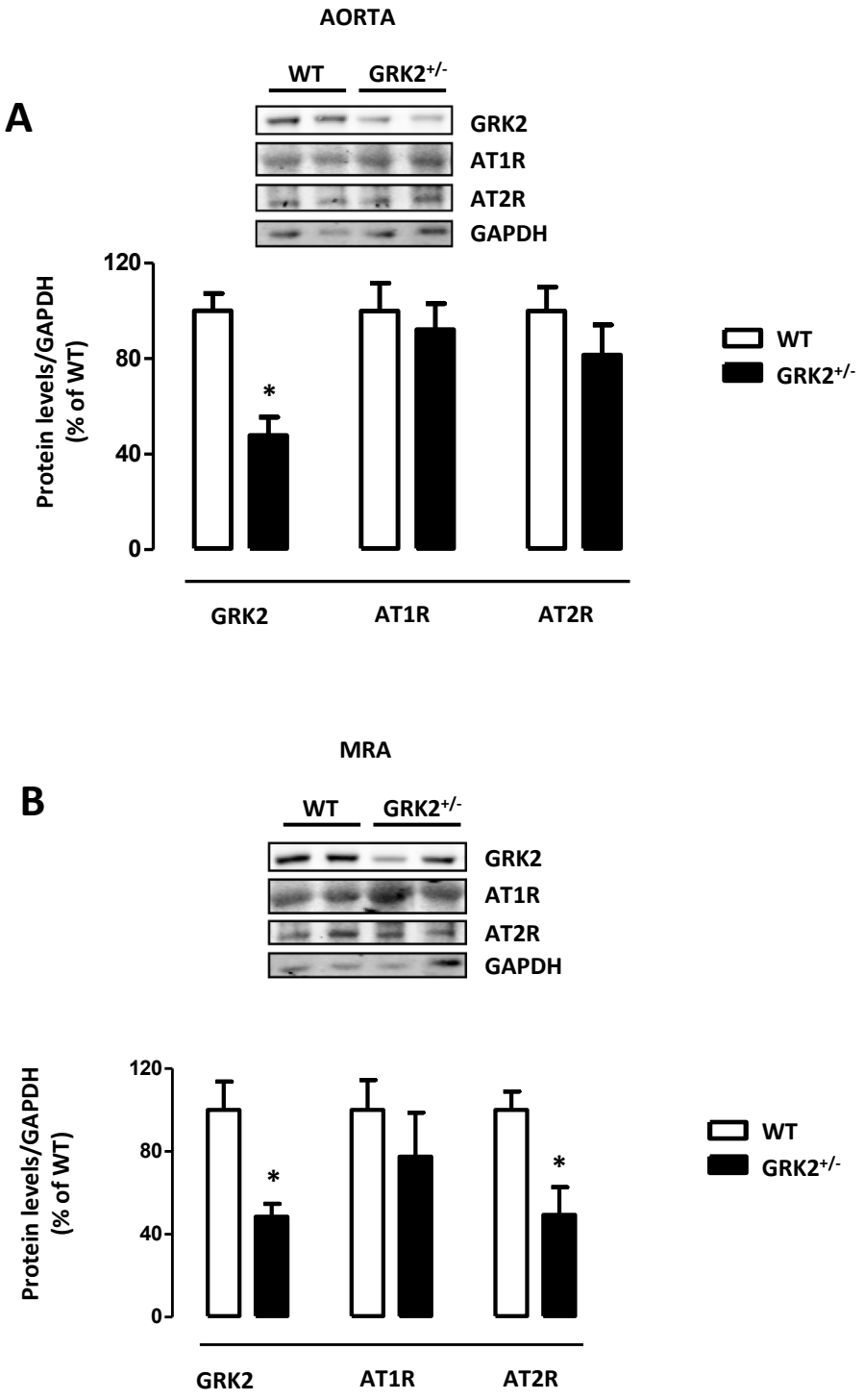

Figure S3. Effects of partial GRK2 deficiency on protein expression of GRK2 and AT1 and AT2 receptors. Representative Western Blot and quantification of the protein expression of GRK2 and AT1 and AT2 receptors (AT1R, AT2R) in aorta (A) and mesenteric resistance arteries (MRA) (B) from WT and GRK2$^{+/-}$ mice. Data were normalized to GAPDH and to values of WT mice that were considered as 100%. n=4-7. *P<0.05 vs WT.

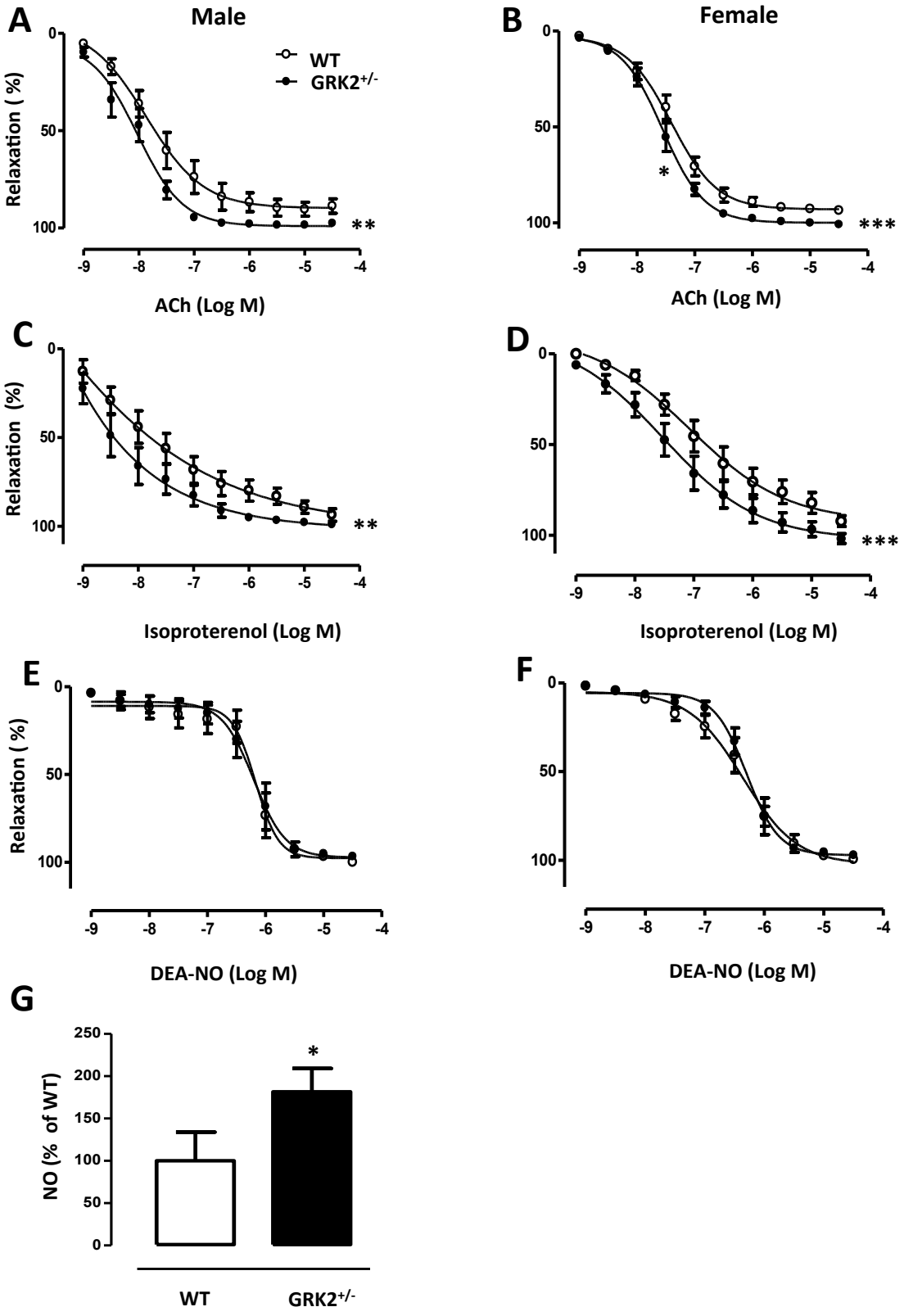

Figure S4. Partial GRK2 deficiency increases vasodilator responses and nitric oxide (NO) production in mesenteric resistance arteries (MRA). Concentration-response curve to acetylcholine (ACh) (A,B), isoproterenol (C,D) and diethylamine NONOate (DEA-NO) (E,F) in MRA from adult male and female wild type (WT) and GRK2$^{+/-}$ mice. (G) Quantification of ACh-induced NO release in MRA from male WT and GRK2$^{+/-}$ mice. n=5-10. *P<0.05, **P<0.01, ***P<0.001.

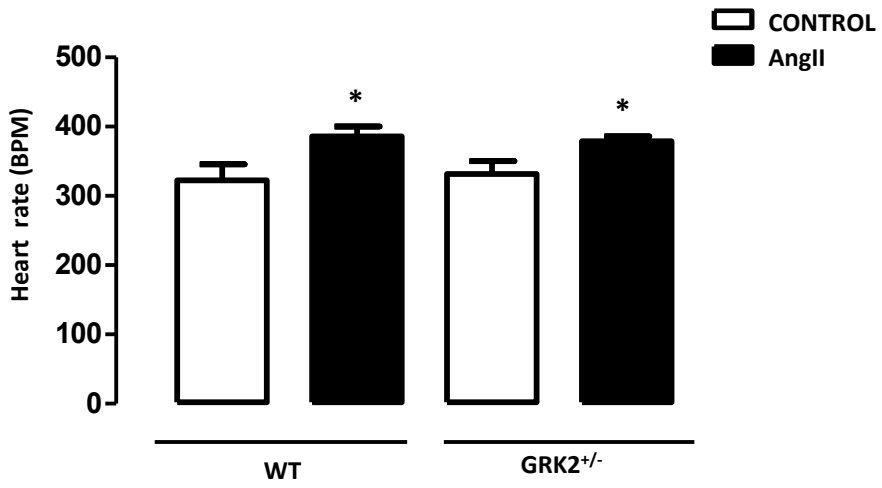

Figure S5. Partial GRK2 deficiency does not modify the effect of Angiotensin II (AngII) infusion on heart rate. Heart rate is expressed as beats per minute (BPM) and was determined in male wild type (WT) and GRK2$^{+/-}$ mice untreated (CONTROL) or treated with AngII. n=5-7. *P<0.05 vs control.

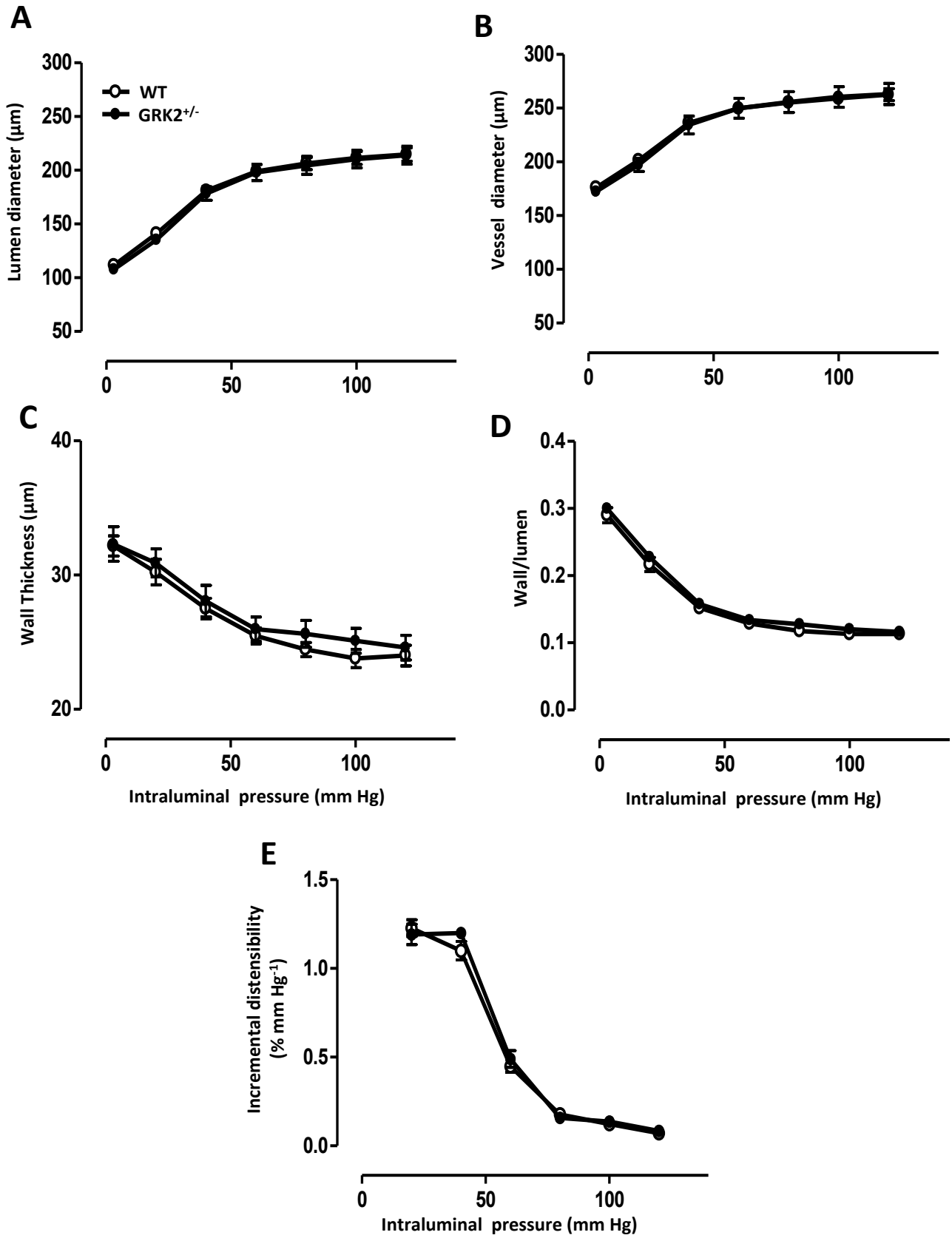

Figure S6. Effects of partial GRK2 deficiency on vascular structural and mechanical parameters. Lumen and vessel diameter-intraluminal pressure (A,B), wall thickness-intraluminal pressure (C), wall/lumen-intraluminal pressure (D), and incremental distensibility-intraluminal pressure (E) in mesenteric resistance arteries incubated in $0Ca^{2+}$Krebs Henseleit solution from wild type (WT) and $GRK2^{+/-}$ mice. n=8-9.

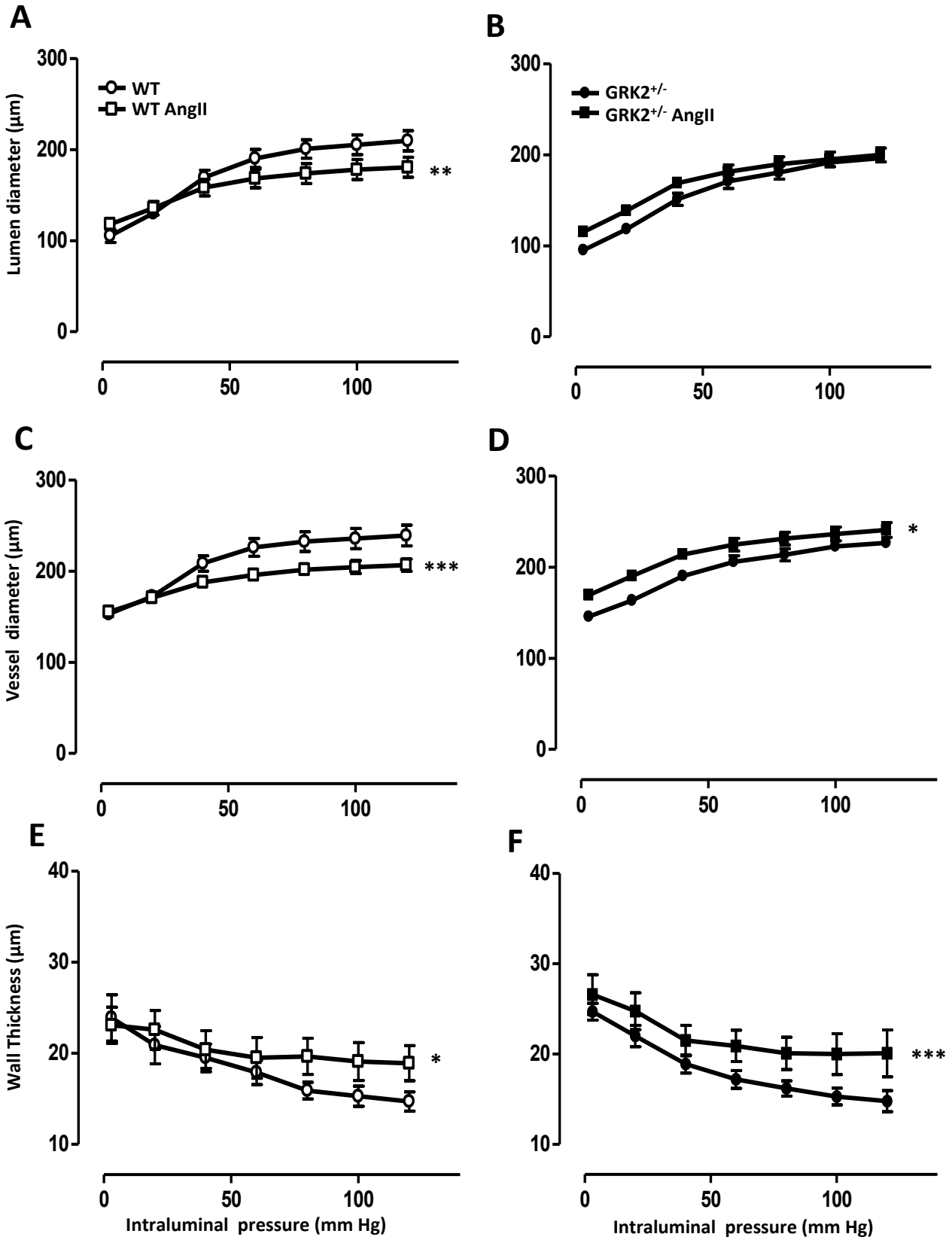

Figure S7. Effects of partial GRK2 deficiency on vascular structural parameters in Angiotensin II (AngII) infused mice. Lumen (A,B) and vessel diameter-intraluminal pressure (C,D) and wall thickness-intraluminal pressure (E,F) in mesenteric resistance arteries incubated in $0Ca^{2+}$Krebs Henseleit solution from wild type (WT) and $GRK2^{+/-}$ mice untreated and treated with AngII. n=5-7. *P<0.05, **P<0.01 , ***P<0.001 vs untreated.

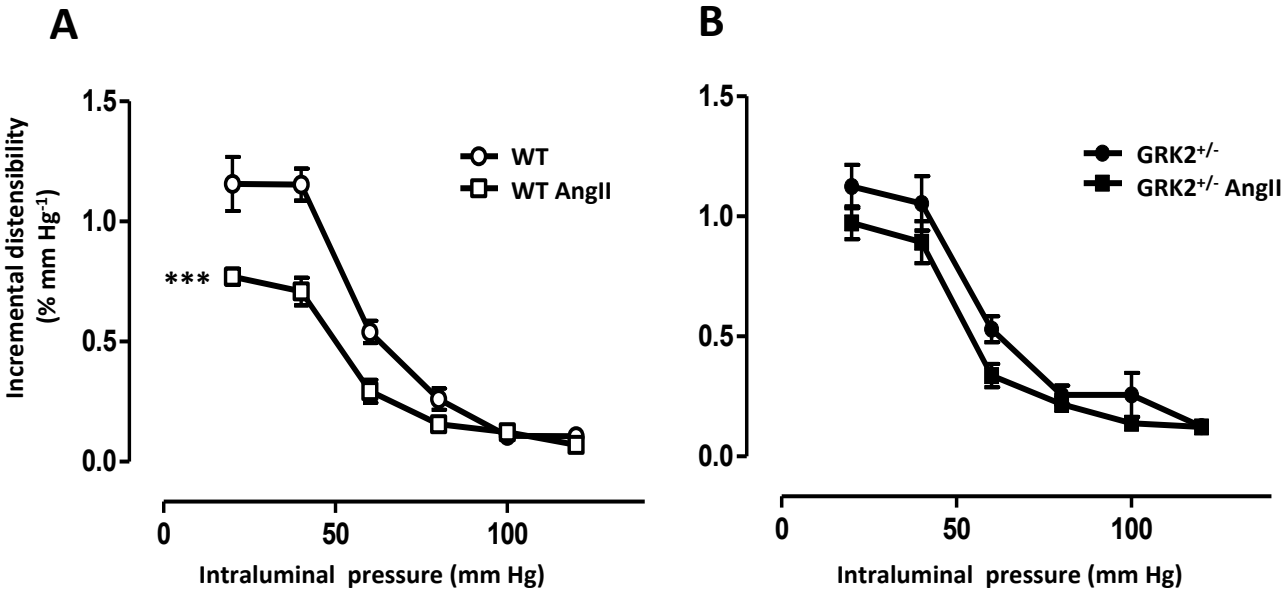

Figure S8. Effects of partial GRK2 deficiency on vascular mechanical parameters in Angiotensin II (AngII)-infused mice. Incremental distensibility-intraluminal pressure in mesenteric resistance arteries incubated in $0Ca^{2+}$Krebs Henseleit solution from wild type (WT) (A) and $GRK2^{+/-}$ (B) mice untreated and treated with AngII. n=5-7. ***P<0.001 vs untreated.

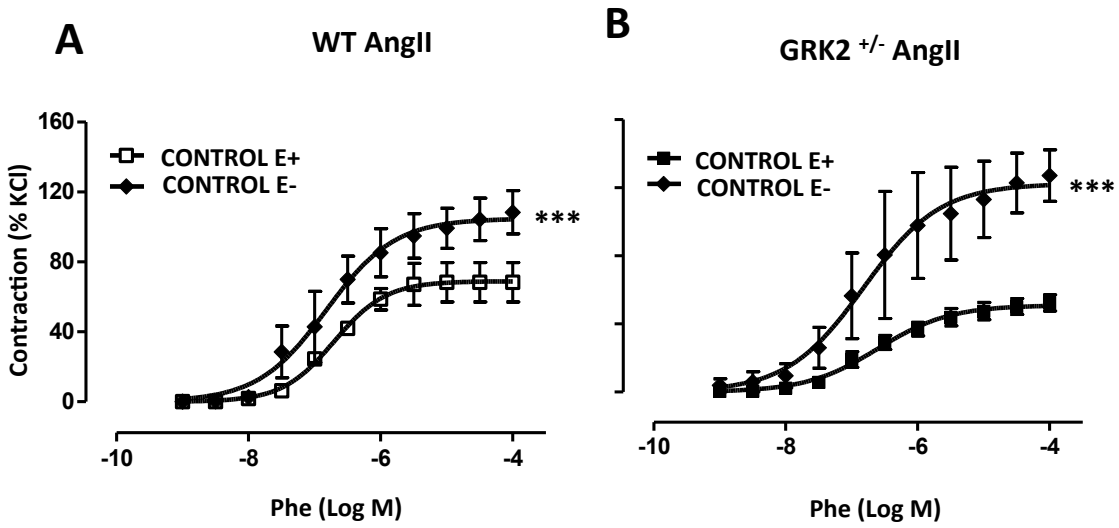

Figure S9. Partial GRK2 deficiency increases endothelial modulation of vasoconstrictor responses. Effect of endothelium removal (E-) on the concentration-response curve to phenylephrine (Phe) in aortic segments from wild type (WT) (A) and GRK2$^{+/-}$ (B) mice treated with Angiotensin II (AngII). Data represent mean±SEM. n=4. *P<0.05, ***P<0.001.

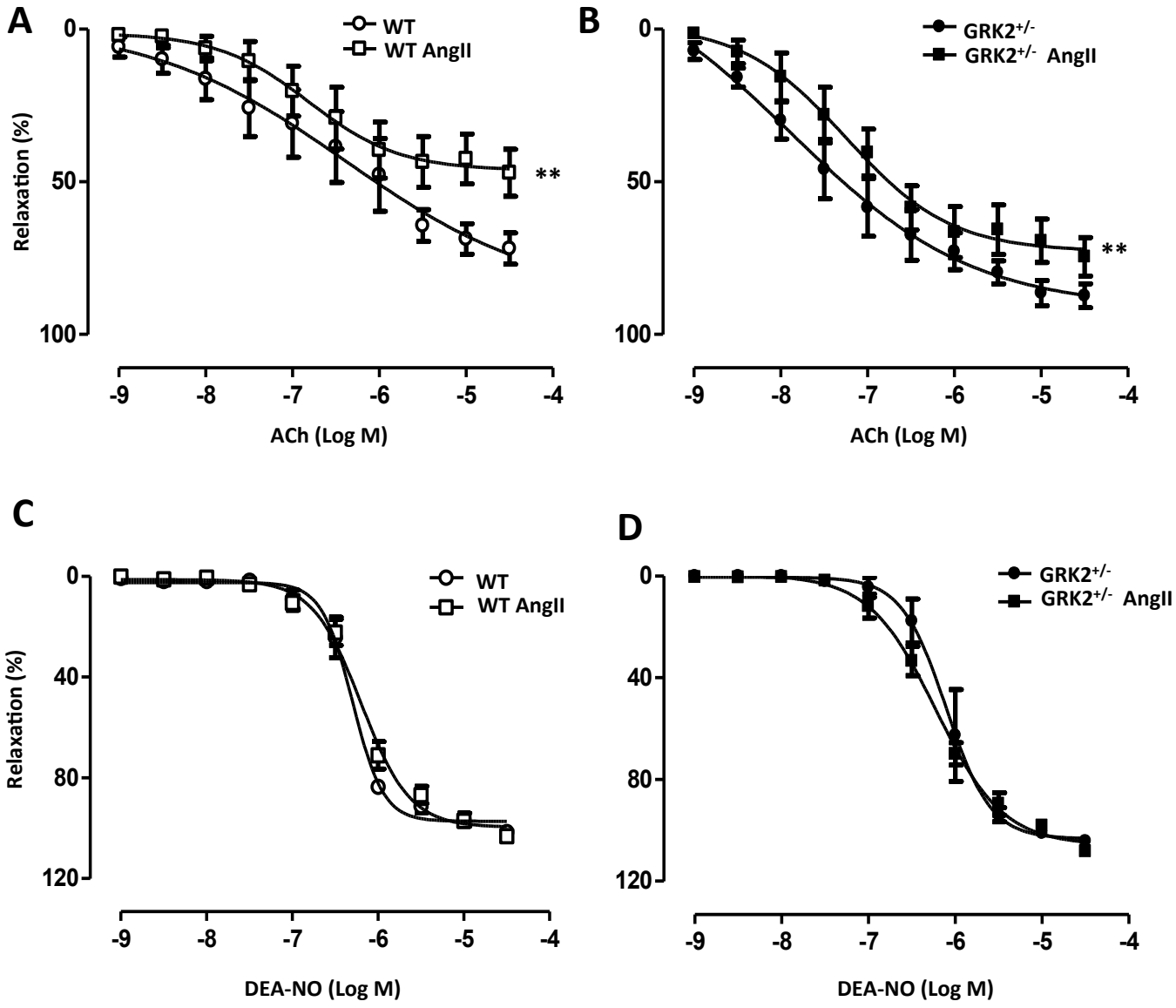

Figure S10. Partial GRK2 deficiency partially prevents Angiotensin II (AngII)-induced endothelial dysfunction but does not affect endothelium independent NO responses. Effect of AngII infusion on the concentration-response curve to acetylcholine (ACh) (A, B) or diethylamine NONOate (DEA-NO) (C, D) in aortic segments from wild type (WT) and GRK2$^{+/-}$ mice. Data represent mean±SEM. n=6-11, **P<0.01.

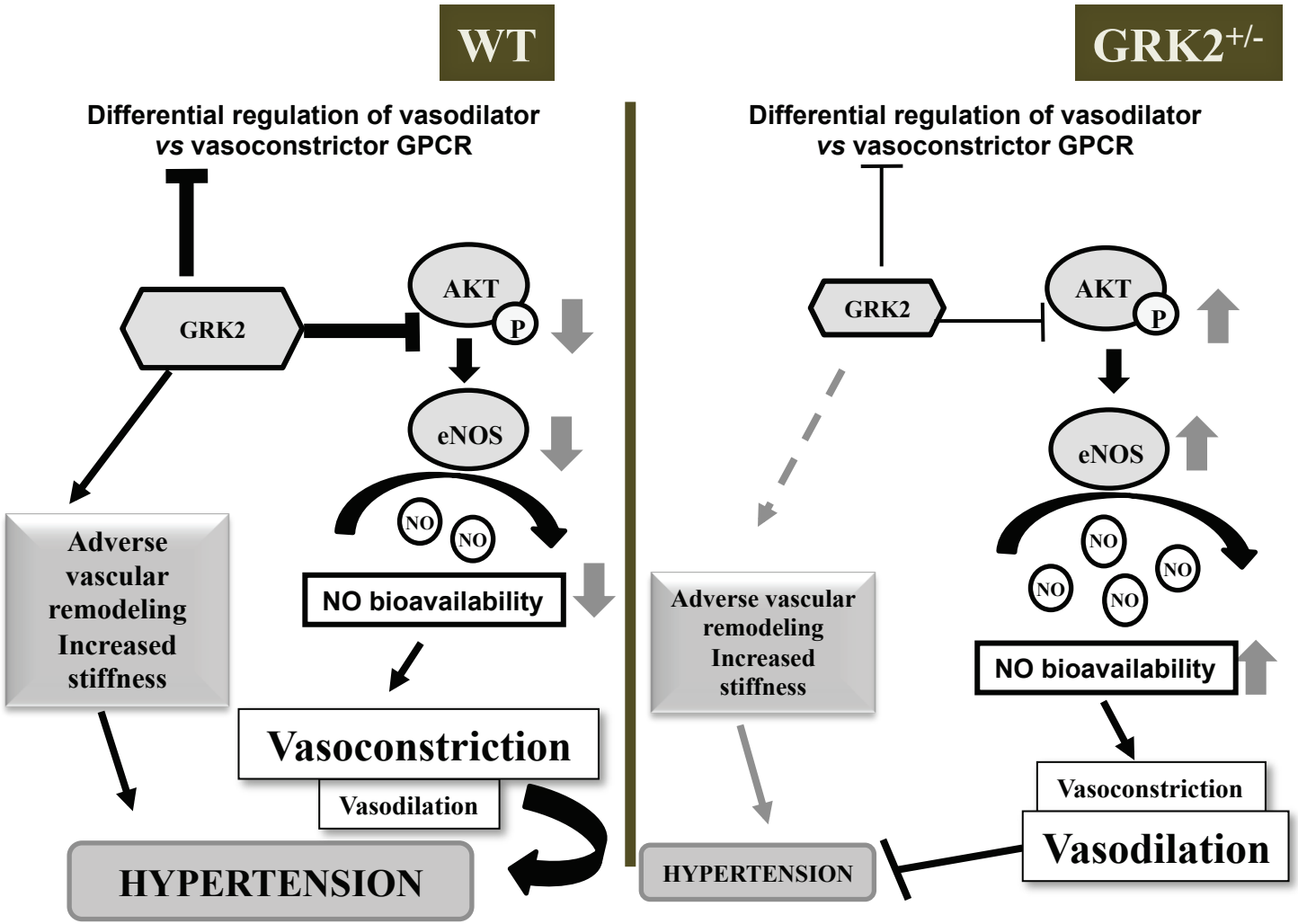

Figure S11. Role of GRK2 in hypertension and vascular responses. GRK2 participates in the regulation of vasoconstrictor and vasodilator G protein-coupled receptors (GPCR). However, GRK2 is also involved in non GPCR-dependent pathways such as the interaction with Akt that inhibits Akt-dependent activation of nitric oxide synthase (NOS) thus impairing nitric oxide (NO) production. Upon Angiotensin II (AngII) challenge, adult GRK2$^{+/-}$ mice show improved vascular remodeling, decreased vascular stiffness, increased Akt activation, less reduced eNOS expression and increased NO production. This provokes decreased vasoconstrictor and increased vasodilator responses what, together with improved vascular structure and mechanics, leads to resistance to severe AngII-induced hypertension.

INTRODUCTION TO ARTICLE #2

The second article presented is the review entitled G Protein-coupled receptor kinase 2 (GRK2): A novel modulator of insulin resistance (Arch Physiol Biochem. 2011; 117 (3):125-30).

This article reviewed the state of the art prior to our study in the heart and highlighted the role of GRK2 in the modulation of insulin signaling in several tissues important for systemic metabolism control, with potential impact and connections in cardiovascular function.

I participated (as second co-author) in the literature search, general organization and structuring of the manuscript, and in reviewing the text. I also prepared the summary figure accompanying the article.

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RESEARCH ARTICLE

G Protein-coupled receptor kinase 2 (GRK2):A novel modulator of insulin resistance

Federico Mayor Jr.[1,2], Elisa Lucas[1,2], María Jurado-Pueyo[1,2], Lucia Garcia-Guerra[3], Iria Nieto-Vazquez[4,6], Rocio Vila-Bedmar[1,2], Sonia Fernández-Veledo[5,6] and Cristina Murga[1,2]

[1]Departamento de Biología Molecular and Centro de Biología Molecular Severo Ochoa (CSIC-UAM), 28049 Madrid (Spain), [2]Instituto de Investigación Sanitaria La Princesa, 28006 Madrid, Spain, [3]Instituto de Investigaciones Biomédicas "Alberto Sols" (CSIC-UAM), Arturo Duperier, 4, 28029 Madrid, [4]Department of Biochemistry and Molecular Biology II, Faculty of Pharmacy, Complutense University, 28040 Madrid, Spain, [5]Hospital Universitari de Tarragona Joan XXIII, IISPV, Universitat Rovira i Virgili, 43007 Tarragona, Spain, [6]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Matabólicas CIBERDEM, Spain

Abstract

G protein-coupled receptor kinase 2 (GRK2) is emerging as a key, integrative node in many signalling pathways. Besides its canonical role in the modulation of the signalling mediated by many G protein-coupled receptors (GPCR), this protein can display a very complex network of functional interactions with a variety of signal transduction partners, in a stimulus, cell type, or context-specific way. We review herein recent data showing that GRK2 can regulate insulin-triggered transduction cascades at different levels and that this protein plays a relevant role in insulin resistance and obesity *in vivo*, what uncovers GRK2 as a potential therapeutic target in the treatment of these disorders.

Key words: G protein-coupled receptors, arrestin, diabetes, obesity

Introduction

G protein-coupled receptor kinases (GRKs) were first described to participate together with other cytoplasmic proteins termed arrestins in the regulation of multiple G protein-coupled receptors (GPCR), a superfamily of membrane sensors with central roles in physiology and as pharmacological targets. GPCR transduce extracellular signals inside the cell through activation of heterotrimeric G proteins, and/or via other G protein-independent signalling pathways more recently identified. Upon agonist binding, GPCR become specifically phosphorylated by GRKs in their intracellular domains (reviewed in Premont & Gainetdinov, 2007; Penela *et al*., 2010). This event promotes the association of arrestins, leading to uncoupling from heterotrimeric G proteins, thus abrogating further G protein-dependent signal propagation. As a result of beta-arrestin binding, phosphorylated receptors are also targeted for clathrin-mediated internalization (DeWire *et al*., 2007).

Phosphorylation of GPCR by GRKs and recruitment of arrestins serves not only to block G protein-dependent signal transduction, but also to initiate alternative signalling pathways, what has become a paradigm shift in the field in the past few years. Arrestins can act as scaffold proteins for several signalling mediators such as c-Src, components of the MAPK cascades, the cAMP phosphodiesterase PDE4, Akt, components of the NFkB signalling pathway or the Mdm2 ubiquitin ligase, among others (Kovacs *et al*., 2009; Luttrell & Gesty-Palmer, 2010). Therefore, arrestin recruitment is critical for triggering the modulation of important intracellular signalling cascades by GPCR, contributing to the overall cellular response to the presence of a messenger.

On the other hand, cellular roles of GRKs are not limited to promoting beta-arrestin binding to activated GPCRs. Seven GRK genes are known in mammals, subdivided into three different groups:visual GRK subfamily

This work is dedicated to the memory of Professor Margarita Lorenzo, who passed away 7 April 2010, at the age of 51.

Address for correspondence:Federico Mayor Jr, Universidad Autónoma Madrid, Centro de Biología Molecular, c/Nicolas Cabrera 1, Universidad Autonoma, Madrid, 28049 Spain. Tel:34-91-196-4626. Fax:34-91-196-4420. E-mail:fmayor@cbm.uam.es

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(GRK1 and GRK7), the beta-adrenergic receptor kinase subfamily (GRK2/GRK3) and the GRK4 subfamily (GRK4, GRK5 and GRK6). Except visual GRKs and GRK4, GRKs are almost ubiquitously expressed, showing different protein levels depending on the tissue and cell type (Premont & Gainetdinov, 2007; Penela *et al*., 2010).

Functionally speaking, GRK2 appears to be the most important isoform as judged by the lethality of homozygous GRK2-deficient mice (Jaber *et al*., 1996). In addition to the regulation of GPCR, this protein is related to the control of several members of the receptor-tyrosine kinase (RTK) family (reviewed in Hupfeld & Olefsky, 2007), phosphorylates a variety of non-receptor substrates and dynamically interacts with other important signal transduction partners in a kinase activity-independent way (see references in Peregrin *et al*., 2006; Ribas *et al*., 2007; Penela *et al*, 2010). The relevance of GRK2 in cell biology and physiology is further stressed by the fact that it participates in basic cellular processes such as differentiation/development (Molnar *et al*, 2007), cell migration (Penela *et al*., 2008), cell cycle progression (Penela *et al*, 2010), and by the fact that its levels are tightly controlled by very complex mechanisms and vary substantially in different pathological situations (Penela *et al*., 2003; Salcedo *et al*., 2006). In this line, changes in GRK2 expression and activity have been described to occur during the onset or progression of several relevant inflammatory and cardiovascular diseases, suggesting that those alterations may contribute to the triggering or development of these pathologies (Penela *et al*. 2006; Dorn, 2009, Vroon *et al*. 2006).

Taking into account these considerations, and in the midst of the aforementioned shift in paradigm (from GRKs being considered as mere negative modulators of GPCR signalling to realizing their more complex role as key nodes of many intracellular transduction pathways) our laboratory started a few years ago a collaboration with the group lead by Margarita Lorenzo to address the potential role for GRK2 in the modulation of insulin signalling. Margarita was a brilliant scientist, full of energy and drive, an excellent professor, an inspiring mentor for her students, and, above all this, a close friend for many years, from the time we both were PhD students at the Universidad Autonoma in Madrid. We really made the most, during this collaborative project, of her insight and profound knowledge of the insulin signalling research field, and enjoyed a lot discussing the exciting data obtained by both laboratories, even when she was already courageously fighting her disease. Margarita leaves all us an enduring scientific legacy and the example of the way she enjoyed both science and life. This review is dedicated to her memory. We will miss and remember her very much.

GRK2 as a modulator of insulin signalling?

Initial works on the characterization of the insulin pathway in 3T3L1 adipocytes reported that insulin-induced GLUT4 translocation required at least two signals in these cells, one mediated through phosphatidylinositol 3-kinase (PI3K) and another via Gαq/11 subunits, and that the activated insulin receptor (IR) could promote Gαq/11 phosphorylation on tyrosine residues, thus leading to activation of cdc42 and PI3K and glucose transport stimulation (Kanzaki *et al*., 2000; Imamura *et al*., 1999; Usui *et al*., 2003). Based on the fact that GRK2 can bind Gαq/11 and prevent its interaction with effectors (Mariggio *et al*., 2007 and references therein), Dr. Olefsky's laboratory showed in 2004 that GRK2 can act as an inhibitor of insulin-mediated glucose transport stimulation in 3T3L1 adipocytes by interfering with Gαq/11 function independently of its kinase activity (Usui *et al*., 2004). Moreover, GRK2 was able to inhibit basal and insulin-stimulated glycogen synthesis in mouse liver FL83B cells (Shahid & Hussain, 2007). In this context, we set out to analyse the modulation of insulin signalling by GRK2 in relevant insulin-targeted cell types. In adipocytes and myoblasts, overexpression of GRK2 or GRK2-K220R (a catalytically inactive kinase mutant) inhibited insulin-stimulated glucose uptake and insulin-dependent signalling (IRS1 tyrosine phosphorylation, Akt and ERK activation) in a kinase-activity independent manner (García-Guerra *et al*., 2010). Conversely, GRK2 silencing enhanced these responses. A similar picture emerged when investigating such parameters *in vivo* in adipose tissue from wild-type or GRK2- hemizygous mice, expressing half the amount of GRK2 protein related to wild type littermates (Jimenez-Sainz *et al*, 2006; Kleibeuker *et al*., 2008). Faster kinetics in insulin-mediated IRS1 and AKT phosphorylation were detected in GRK2± mice in insulin-responsive tissues *in vivo* (García-Guerra *et al*., 2010).

Interestingly, we observed that basal IRS1 protein levels were increased in cells in which GRK2 had been silenced, and less IRS1 protein was found in cells with elevated kinase dosage, suggesting the existence of a functional relationship that co-ordinately regulates the expression of these two proteins. It was previously shown that enhanced GRK2 levels favour insulin resistance induced either by endothelin-1 in 3T3L1 cells (Usui *et al*., 2005) or by chronic β-adrenergic receptor stimulation in HEK-293 cells (Cipolletta *et al*., 2009). GPCR agonists promote GRK2 interaction with Gαq and also with IRS1, resulting in decreased insulin-stimulated glucose transport, in IRS1 inhibitory phosphorylation at serine residues, and in IRS1 degradation. Our data in cultured cells as well as in adipose tissue in vivo show that IRS1 levels as well as the amount of basal GRK2-IRS1 complexes depend on the levels of GRK2 expression, and that insulin stimulation rapidly disrupts basal IRS1/GRK2 complexes, a process that is hampered in situations where the levels of GRK2 are elevated (García-Guerra *et al*., 2010).

The mechanisms modulating this dynamic GRK2/IRS1 association in physiological conditions and the potential implication of phosphorylation events on GRK2-mediated IRS1 modulation are key issues for future research. Control

of cellular IRS1 expression levels has been described to involve proteasome-mediated degradation, phosphatase-mediated dephosphorylation (Boura-Halfon & Zick, 2009), and phosphorylation of IRS1 on serine residues, what also reduces the ability of IRS1 to associate with the insulin receptor and thereby inhibits downstream signalling and insulin action (see references in Nieto-Vazquez *et al*., 2008). It was recently described that basal and insulin-stimulated Ser307 phosphorylation of IRS1 was significantly decreased in GRK2-deficient liver FL83B cells (Shahid & Hussain, 2007). Also, Usui *et al*. (2005) and Cipolletta *et al* (2009) have proposed that GRK2 mediates endothelin-1 and adrenergic-induced insulin resistance respectively by enhancing IRS1 serine phosphorylation and degradation. Whether this is a direct or an indirect effect of GRK2/IRS1 association is not straightforward. A catalytically inactive GRK2 mutant is able to inhibit insulin-triggered glucose transport and signalling (Usui *et al*., 2004; García-Guerra *et al*., 2010) and leads to decreased IRS1 levels in adipocytes and myocytes (García-Guerra *et al*., 2010), whereas Cipolleta *et al*. (2009) have suggested that IRS1 is a direct target of GRK2 kinase activity in HEK-293 cells. These authors also report, in apparent discrepancy with our data, an increased GRK2-IRS1 co-precipitation after 30 minutes of insulin treatment in this cell type and in tissues from spontaneously hypertensive SHR rats. These discrepancies may arise from the different cell source used or reflect a more complex nature of the GRK2/IRS1 dissociation/association/phosphorylation dynamics upon insulin receptor and/or GPCR activation than previously anticipated.

In sum, the available data suggest that altered GRK2 levels could lead to modulation of insulin signals through GRK2-Galphaq/11 binding, GRK2-IRS1 association and/or phosphorylation, modulation of IRS1 stability, or altered GPCR-mediated transmodulation of the insulin pathway engagement (Figure 1). The precise contribution of such mechanisms to different physiological and pathological conditions remains to be investigated in detail in the future.

GRK2 plays a key role in the modulation of insulin resistance and obesity *in vivo*

Insulin resistance, a diminished ability of cells to respond to the action of insulin, is a key feature associated with the pathogenesis of metabolic disorders such as type-2-diabetes and obesity (Biddinger & Kahn, 2006). Alterations in any of the key components of the insulin signaling cascade, including negative regulators, have been proposed to contribute to insulin resistance. However, the full complexity underlying the origin and the mechanism(s) that mediate insulin resistance in different physiopathological conditions are not completely understood (Biddinger & Kahn, 2006; Hoehn *et al*., 2008).

We reasoned that if GRK2, as an established negative modulator of insulin signalling in cellular models (see above), was to play a physiological role in the modulation of insulin sensitivity in vivo, two conditions should be met:(a) the expression of this kinase should be altered in situations characterized by insulin resistance; (b) lowering GRK2 levels should protect against the induction of systemic insulin resistance.

Interestingly, we observed that GRK2 expression was enhanced by approximately 2-fold in insulin-resistant cultured human adipocytes, in white adipose tissue of the ob/ob model of obese mice, and in adipose and muscle tissues of either TNFα-, age- or high fat diet-induced insulin resistance mice models (García-Guerra *et al*., 2010). In addition, since lymphocytic GRK2 levels have been reported to mirror changes in kinase expression in other organs under several physiopathological circumstances (Iaccarino *et al*., 2005), we analysed the levels of GRK2 in peripheral blood mononuclear cells obtained from

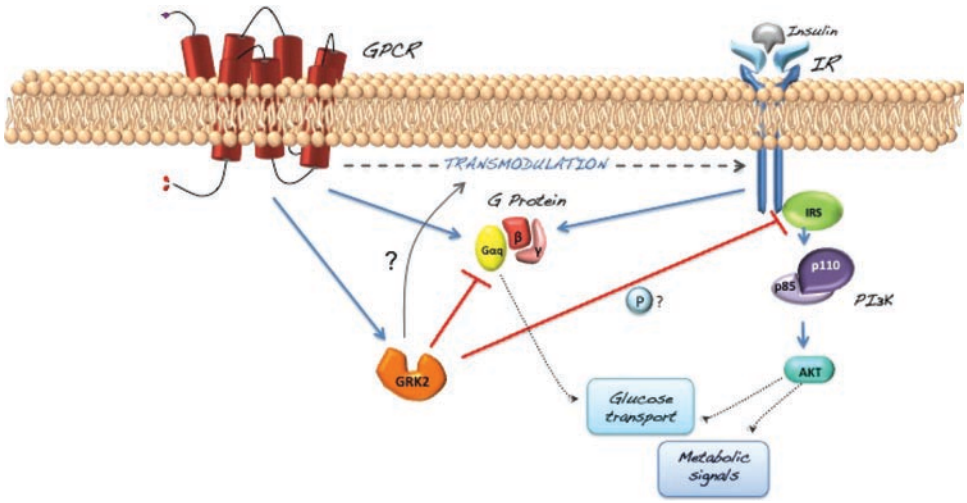

Figure 1. Insulin acts by binding to its receptor (IR) which recruits insulin receptor substrate proteins (IRS) to activate the phosphatidylinositol 3-OH kinase (PI3K)/Akt pathway, or alternatively heterotrimeric Gq proteins (G protein) in both cases leading to glucose uptake and metabolic regulation. GRK2 protein can bind to Gq or to IRS, thereby impairing downstream insulin signals. On the other hand, G protein-coupled receptors can transmodulate insulin pathway signals and this crosstalk could also be regulated by GRK2 levels.

patients with diverse degrees of insulin resistance. We found that GRK2 levels were higher in insulin-resistant patients compared to control individuals (García-Guerra *et al.*, 2010). Consistent with our findings, enhanced GRK2 expression has also been reported in tissues from obese Zucker rats (Trivedi *et al.*, 2005). Overall, these results are consistent with GRK2 as an important player of the process leading to insulin-resistant conditions *in vivo*.

Importantly, we found that GRK2 haploinsufficient mice maintain glucose tolerance and insulin signalling in the major insulin-responsive tissues under either TNFα, ageing or high fat diet conditions (García-Guerra *et al.*, 2010), a phenotype reminiscent to that of mice deficient in other well established negative regulators of insulin signalling such as PTP1B (Nieto-Vázquez *et al.*, 2007 and references therein). These data support the notion that GRK2 can be regarded as a *bona fide* novel negative regulator of insulin action. The fact that a 50% down-regulation of kinase levels in hemizygous GRK2± mice is sufficient to protect against the induction of insulin resistance strongly suggests that enhanced GRK2 expression, at least above a certain threshold, would markedly impair insulin signalling *in vivo*, and argues for a key role for GRK2 in the modulation of insulin sensitivity in physiological and pathological conditions.

The results obtained to date raise an important question to be addressed in future studies, namely the identification of the mechanisms and stimuli that trigger enhanced GRK2 expression under insulin-resistant conditions. We have previously shown in other cell types that activation of the PI3K/AKT pathway by IGF-1 increases GRK2 levels (Salcedo *et al.*, 2006), and also chronic insulin has been reported to increase GRK2 levels in HEK-293 and liver FL83B cells (Cipolleta *et al.*, 2009; Shahid & Hussain, 2007). Thus, one possible mechanism for increased GRK2 levels during insulin resistance is that the hyperinsulinemia associated to these conditions (and clinically to obesity and type-2 -diabetes) would trigger the observed up-regulation of GRK2. In this context, it is interesting to note that β-arrestin-2, another protein closely related to GRKs in their canonical function as GPCR regulators, has been recently put forward as a positive regulator of insulin sensitivity, by serving as a scaffold of AKT and Src to the IR (Luan *et al.*, 2009). It is tempting to speculate that enhanced GRK2 levels could also interfere insulin signalling by altering the recently described IR-arrestin functional interface, either by re-directing arrestin to other partners, such as GPCR or, given that GRK2 can directly interact with AKT or Src (Sarnago *et al*, 1999; Liu *et al.*, 2005), by GRK2-dependent inhibition of the formation of the arrestin/AKT/Src complexes required for efficient insulin signalling.

In animal models and in human patients with insulin resistance, β-arrestin2 levels are downregulated (Luan *et al.*, 2009). Therefore, it is tempting to suggest that the combined effects of an up-regulation of GRK2 and a down-regulation of β-arrestin2 in insulin-resistant conditions could lead to major alterations in insulin signalling pathways and in GPCR-IR cross-talk. In this context, it is worth noting that elevated levels of insulin and IGF-1 have been shown to exert differential effects on GRK2 and β-arrestin2 expression (Dalle *et al.*, 2002; Hupfeled *et al.*, 2003; Salcedo *et al.*, 2006). The role of hyperinsulinemia in modulating both GRK2 and beta-arrestin expression in different relevant cell types is being actively investigated in our laboratory.

Alternatively, or in addition, an altered cytokine expression pattern, typical of insulin-resistant states, could also contribute to enhance GRK2 expression levels in these circumstances. TNFα or IL-6 have been reported to increase the expression of other negative modulators of the insulin signalling cascade like PTP1B (Nieto-Vázquez *et al.*, 2007 and 2008), and we found that TNFα infusion also enhances GRK2 protein content in adipose tissue and muscle of wild-type mice in the presence of mild hyperinsulinemia (García-Guerra *et al.*, 2010). Pro-inflammatory mediators can modulate endogenous GRK2 expression in a cell-type specific fashion (Ramos-Ruiz *et al.*, 2000; Eijkelkamp *et al.*, 2010), and an increase in GRK2 protein is found upon chronic IL-1beta treatment in astrocytes (Kleibeuker *et al.*, 2008). Interestingly, GRK2 appears to have a relevant role in the aetiology and/or in the development of several inflammatory diseases (Vroon *et al.*, 2006), so the role for GRK2 in states of insulin resistance associated to inflammatory diseases deserves further investigation.

GRK2 levels as modulators of age- and diet-induced adiposity

It is becoming increasingly clear that impaired insulin actions in adipose tissue are key to the overall insulin resistance characteristic of adult and/or obese animals. Adipocyte-conditioned medium impairs insulin signalling in muscle cells and hepatocytes, and mice that exhibit reduced adiposity typically display improved glucose tolerance and increased insulin sensitivity (Guilherme *et al.*, 2008; Sabio *et al.*, 2008; Fernandez-Veledo *et al.*, 2008). On the other hand, GRK2 hemizygous mice subjected to a HFD gain less weight and have smaller adipocytes compared with wild type littermates, and 9 month-old hemizygous animals display reduced adiposity and lower circulating levels of insulin and leptin than wild type controls (García-Guerra *et al.*, 2010). Therefore, it is possible that part of the insulin hypersensitivity detected in GRK2± mice and the protection afforded to the development of insulin resistant conditions in these animals is related to the observed changes in adiposity. Further analysis of the specific role of GRK2 in muscle tissue, the mechanisms of GRK2 in regulating adipogenesis and the use of cell type/tissue-specific GRK2 down-regulation models will provide new insights into the role of GRK2 in obesity and insulin resistance.

Concluding remarks

Recently published data add to those already present in the literature to support the identification of GRK2 as an important negative regulator of insulin effects, key to the etiopathogenesis of insulin resistance and obesity, thus uncovering this protein as a potential therapeutic target in the treatment of these disorders. A sustained systemic inhibition of kinase functionality could be recapitulated in adult GRK2± mice. Since these animals do not develop insulin resistance in response to a variety of triggers of this condition, it could be hypothesized that sustained GRK2 inhibition may constitute a good therapeutic strategy against these disorders. In fact, one study in animal models of type-2-diabetes has shown that GRK2 inhibition through systemic delivery of small peptides derived from its catalytic domain results in improved glucose homeostasis (Anis *et al.*, 2004). However, it remains to be established whether inhibition of GRK2 prior or during the development of insulin resistance is required for an amelioration of the condition or, alternatively, whether a GRK2 reduction can have a therapeutic effect even after insulin resistance is overtly established. Also, from a mechanistic point of view, it will be interesting to study whether inhibition of catalytic activity, down-regulation of protein expression or targeted disruption of the specific interaction of GRK2 with insulin signalling pathway components are the more appropriate strategies for specifically improving insulin sensitivity.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the article.

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INTRODUCTION TO ARTICLE #3

The third article presented herein was entitled Downregulation of G protein-coupled receptor kinase 2 levels enhances cardiac insulin sensitivity and switches on cardioprotective gene expression patterns (Biochim Biophys Acta. Molecular Basis of Disease 2014; 1842:2448–2456).

The aim of this work was to investigate in detail the interconnections among GRK2 dosage, cardiac insulin sensitivity, cardiac gene expression patterns and heart phenotype in adult mice or animals fed with high-fat diet, two conditions known to promote insulin resistance.

This is the first study to indicate a cardioprotective role for lowering GRK2 levels in adult 9 month-old mice and the first to report alterations in cardiac GRK2 levels under different insulin-resistance conditions such as HFD feeding and ob/ob animals.

Building on data obtained by Dr. María Jurado Pueyo´s in her Doctoral Thesis, this work characterized the activation of insulin signaling pathways, tissue remodeling in the heart and cardioprotective gene expression patterns in young and adult WT and GRK2+/− mice. In addition, we investigated the effect of systemic insulin-resistance-promoting conditions such as high-fat diet feeding on cardiac GRK2 levels and its impact on cardiac insulin responses. Our data point at new molecular links between GRK2 up-regulation in insulin-resistance-related situations and maladaptive cardiac remodeling in the adult mouse heart.

As first shared co-author of this work, I performed the study of insulin signaling cascade activation in both WT and GRK2+/- adult mice, contributed to the study of GRK2 alterations under insulin resistance conditions and its implications for cellular signaling and corroborated the protective role of lowering GRK2 levels under diet-induced insulin resistance conditions. The initial gene microarray data as well as the morphological and physiological analysis of adult cardiac tissue was part of Dr. María Jurado´s thesis, while I performed gene expression validation experiments and further analysis of the results obtained from the arrays.

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Downregulation of G protein-coupled receptor kinase 2 levels enhances cardiac insulin sensitivity and switches on cardioprotective gene expression patterns

Elisa Lucas [a,b,1], María Jurado-Pueyo [a,b,1], María A. Fortuño [c], Sonia Fernández-Veledo [d], Rocío Vila-Bedmar [a,b], Luis J. Jiménez-Borreguero [b,e], Juan J. Lazcano [e], Ehre Gao [f], Javier Gómez-Ambrosi [g], Gema Frühbeck [g], Walter J. Koch [f], Javier Díez [c,h], Federico Mayor Jr. [a,b,*], Cristina Murga [a,b,**]

[a] Departamento de Biología Molecular and Centro de Biología Molecular Severo Ochoa (UAM-CSIC), Madrid, Spain
[b] Instituto de Investigación Sanitaria La Princesa, Madrid, Spain
[c] Division of Cardiovascular Sciences, Centre for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain
[d] Hospital Universitari de Tarragona Joan XXIII, IISPV, Universitat Rovira i Virgili, CIBERDEM, Spain
[e] Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain
[f] Department of Pharmacology and Center for Translational Medicine, Temple University, Philadelphia, USA
[g] Metabolic Research Laboratory, Universidad de Navarra, CIBERobn, Pamplona, Spain
[h] Department of Cardiology and Cardiovascular Surgery, University Clinic, University of Navarra, Pamplona, Spain

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ABSTRACT

G protein-coupled receptor kinase 2 (GRK2) has recently emerged as a negative modulator of insulin signaling. GRK2 downregulation improves insulin sensitivity and prevents systemic insulin resistance. Cardiac GRK2 levels are increased in human heart failure, while genetically inhibiting GRK2 leads to cardioprotection in mice. However, the molecular basis underlying the deleterious effects of GRK2 up-regulation and the beneficial effects of its inhibition in the heart are not fully understood. Therefore, we have explored the interconnections among a systemic insulin resistant status, GRK2 dosage and cardiac insulin sensitivity in adult (9 month-old) animals. GRK2$^{+/-}$ mice display enhanced cardiac insulin sensitivity and mild heart hypertrophy with preserved systolic function. Cardiac gene expression is reprogrammed in these animals, with increased expression of genes related to physiological hypertrophy, while the expression of genes related to pathological hypertrophy or to diabetes/obesity co-morbidities is repressed. Notably, we find that cardiac GRK2 levels increase in situations where insulin resistance develops, such as in ob/ob mice or after high fat diet feeding. Our data suggest that GRK2 downregulation/inhibition can help maintain cardiac function in the face of co-morbidities such as insulin resistance, diabetes or obesity by sustaining insulin sensitivity and promoting a gene expression reprogramming that confers cardioprotection.

1. Introduction

G protein-coupled receptor kinases (GRKs) were initially identified as serine–threonine kinases able to phosphorylate agonist-activated G protein-coupled receptors (GPCRs), triggering the binding of arrestins to the receptor, which impairs G protein coupling in a process known as desensitization [1]. However, very recent findings suggest that the GRK2 isoform is also a key controller of insulin receptor signaling [2]. GRK2 downregulation can prevent the development of metabolic disorders, modulating energy expenditure and brown fat function [3] and also insulin actions in peripheral tissues [2]. Notably, GRK2$^{+/-}$ mice (expressing 50% less protein than control littermates) show improved systemic insulin sensitivity, display enhanced activation of the insulin-mediated Akt pathway in muscle, adipose tissue and liver, and are resistant to the induction of insulin resistance (IR) in three different mouse models of this condition [2]. Remarkably, such differences in insulin sensitivity between wild type (WT) and GRK2 hemizygous mice were noted in the adult stage but were not evident in young animals [2].

GRK2 has also been described to play a relevant role in cardiovascular physiopathology. Increased cardiac GRK2 levels have been reported in

Abbreviations: GPCR, G protein-coupled receptor; GRK2, G protein-coupled receptor kinase 2; IR, insulin resistance; HF, heart failure; HFD, high fat diet; WT, wild type

* Correspondence to: F. Mayor Jr., Centro de Biología Molecular Severo Ochoa, c/Nicolás Cabrera, 1, Universidad Autónoma, 28049 Madrid, Spain. Tel.: +34 91 1964626; fax: +34 91 196 4420.
** Correspondence to: C. Murga, Centro de Biología Molecular Severo Ochoa, c/Nicolás Cabrera, 1, Universidad Autónoma, 28049 Madrid, Spain. Tel.: +34 91 1964641; fax: +34 91 196 4420.
E-mail addresses: fmayor@cbm.uam.es (F. Mayor), cmurga@cbm.uam.es (C. Murga).
[1] Equal contribution to this work.

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patients with ischemic or idiopathic dilated cardiomyopathy, cardiac ischemia, hypertension, volume overload and left ventricular hypertrophy [1,4,5]. Also, enhanced GRK2 expression induced by neurohormonal activation has been associated with lower cardiac function and poorer prognosis in human heart failure (HF) and appears as an early event in maladaptive cardiac remodeling in HF [5–7], altogether putting forward GRK2 as a relevant therapeutic target in this myocardial disease (reviewed in ref. [6]). Consistently, genetic inhibition of GRK2 is cardioprotective in different animal models ([1,5–8] and the references therein), and hemizygous GRK2 mice are hyper-responsive to catecholamines and display enhanced cardiac contractility and function, whereas transgenic mice overexpressing different levels of this kinase show an impaired adrenergic cardiac response [9]. However, the detailed molecular mechanisms and the relevant functional interactions underlying the deleterious effects of elevated GRK2 levels in cardiac function and the beneficial effects of its inhibition remain to be fully established.

In principle, up-regulation of GRK2 in HF would further exacerbate the marked β-adrenergic desensitization observed in such condition. However, chronic adrenergic activation appears to be more detrimental than beneficial for heart disease and, most importantly, β-blockers represent a successful standard treatment for this disease. In this context, the fact that GRK2 inhibition acts in a synergic manner with established β-blocker treatments (reviewed in [6,7]) suggests that these two therapeutic strategies must have independent mechanisms of action. Thus, the functional impact of altered GRK2 levels might also be related to the interactions of this protein with cellular partners other than β-adrenergic receptors [10].

In this regard, adding to the data showing that GRK2 up-regulation inhibits insulin signaling in muscle or adipose tissue [2], recent findings have suggested that cardiac-specific overexpression of GRK2 inhibits glucose uptake and promotes IR after myocardial ischemia in young mice [11]. However, the potential interconnections among a systemic IR status, GRK2 levels and the cardiac maladaptive remodeling linked to cardiac dysfunction have not been addressed to date.

In this report, we have characterized the activation of insulin signaling pathways, tissue remodeling in the heart and cardioprotective gene expression patterns in young and adult WT and GRK2$^{+/-}$ mice. This experimental model allows us to explore the consequences of a chronic, physiological-range change in GRK2 levels with age (a risk factor for the onset of most cardiovascular pathologies) that would mimic the long sought pharmacological systemic inhibition of GRK2 as a potential drug target. In addition, we have investigated the effect of systemic IR-promoting conditions on cardiac GRK2 levels and its impact on cardiac insulin responses. Our data point at new molecular links between GRK2 up-regulation in IR-related situations and maladaptive cardiac remodeling in the adult mouse heart.

2. Materials and methods

2.1. Animals

Experiments were performed on male wild type and hemizygous-GRK2 (GRK2$^{+/-}$) mice maintained on the hybrid 129/SvJ C57BL/6 background. The animals were bred and housed on a 12-hour light/dark cycle with free access to food and water. GRK2$^{+/-}$ mice, as well as male leptin-deficient obese *ob*/*ob* mice (C57BL/6J-*Lep*^{ob}/*Lep*^{ob}) and their corresponding wild types (C57BL/6J, The Jackson Laboratory, Bar Harbor, ME, USA) were fed ad libitum since weaning on either a normal chow diet (providing 13% of total calories as fat, 67% as carbohydrate and 20% as protein; 2014S Rodent Maintenance Diet, Teklad, Harlan, Barcelona, Spain) or a high fat diet (providing 45% of total calories as fat, 35% as carbohydrate and 20% as protein, Rodent Diet D12451, Research Diets, New Brunswick, NJ, USA). Animals were maintained at a room temperature of 22 ± 2 °C on a 12:12 light–dark cycle (lights on at 08:00 am) with a relative humidity of 50 ± 10% and under pathogen-free conditions. All animal experimentation procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals (Directive 86/609) and approved by the Ethical Committees for Animal Experimentation of the Universidad Autonoma de Madrid and the University of Navarra (protocols 013/08 and 041/08).

2.2. Plasma membrane preparation and GLUT4 translocation quantification

Relative quantification of GLUT4 protein in the plasma membrane fraction was achieved by a subcellular fractionation method modified from Rett K. et al. [12]. Mice were injected intravenously in the tail vein with insulin (1 unit/kg of body weight). After 25 min, mice were sacrificed by cervical dislocation and hearts were homogenized using metal beads in a Tissue Lyser using cooled buffer (200 mM Tris–HCl pH 7.5, 10 mM EDTA, 255 mM Sucrose) with protease inhibitors (100 μM PMSF, 1 μM benzamidine, 10 μg/ml STI, 16 μU aprotinin, 10 μg/ml bacitracin). Lysates were centrifuged at 9000 ×*g* for 20 min. The pellet (P1) was resuspended in buffer, homogenized again and centrifuged at 200 ×*g* for 20 min. The plasma membrane-enriched P2 was then resuspended and homogenized in RIPA buffer (100 mM Tris–HCl pH 7.4, 600 mM NaCl, 2% Triton X-100, 0.2% sodium dodecyl sulfate, 1% deoxycholate plus protease inhibitors) for Western Blot analysis. Protein content was quantified using the Lowry procedure and 40 μg of total protein was resolved on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. Blots shown here were probed with specific antibodies against GLUT4 (EMD Millipore, Darmstadt, Germany), Caveolin 3 (BD Transduction Laboratories, Franklin Lakes, NJ, USA) and α-Tubulin (Santa Cruz Biotechnology, Dallas, TX, USA).

2.3. Insulin treatments and Western Blots

Insulin (Actrapid®, Novo Nordisk, Bagsvaerd, Denmark) solution in saline serum (1 unit/kg body weight) was administered by tail vein injection for acute cardiac insulin pathway activation analysis. After either 3 or 5 min, mice were sacrificed by cervical dislocation and hearts were quickly collected, washed and frozen at −70 °C. Heart tissue was homogenized using metal beads in a Tissue Lyser using hypotonic buffer (20 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM EGTA) completed with protease and phosphatase inhibitors (100 μM PMSF, 1 μM benzamidine, 10 μg/ml STI, 16 μU aprotinin, 10 μg/ml bacitracin and Phosphatase Inhibitor Cocktail—PhosSTOP (Roche, Indianapolis, IN, USA)—following manufacturer's protocol). Then 0.1% (v/v) Triton X-100 was added and the samples were agitated for 1 h at 4 °C, and centrifuged to measure supernatant protein content using the Lowry procedure. For Western Blot analysis, typically 40 μg of total cardiac protein was resolved per lane on a 7.5% SDS-PAGE gel and transferred to a nitrocellulose membrane. Blots were probed with specific antibodies against phospho-Akt (Ser473), Akt, phospho-p70S6K (Thr389), phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology, Beverly, Ma, USA), p70S6K, phospho-IRS1 (Tyr896) (BD Biosciences, Franklin Lakes, NJ, USA), ERK-1, ERK-2, GRK2, Nucleolin (Santa Cruz Biotechnology, Dallas, TX, USA), GAPDH (Abcam, Cambridge, UK) and IRS1-PH (kindly provided by Deborah J. Burks, Spain) [13].

2.4. Cardiac morphometry

For the histological and morphometric analysis of the hearts, mice were anesthetized with isoflurane and an intracardiac injection of cold KCl solution was used to stop the heart in diastole before the heart collection. Hearts were weighted for cardiac index determination, and the medial section was fixed in formaldehyde and embedded in paraffin prior to transversal sectioning using a microtome. Masson's Trichrome staining was performed for the morphometric analysis and Syrius red staining for fibrosis quantification. Images were analyzed using the AnalySIS® software (Soft Imaging System).

2.5. Echocardiography

To measure global systolic cardiac function and left ventricular mass (LVM), echocardiography was performed in 9 month-old WT and hemizygous GRK2 mice. Mice were anesthetized by inhalation of isoflurane/oxygen (1.25%/98.75%) and examined by a 30 MHz transthoracic echocardiography probe. Images were obtained with Vevo 770 (VisualSonics, Toronto, Canada). The internal diameter of the LV was measured in the short-axis view from M-mode recordings in end diastole and end systole and ejection fraction (EF) and fractional shortening (FS) were calculated using the formulas as previously described [14].

2.6. RNA isolation and microarray analysis

mRNA from frozen heart tissue was extracted using metal beads (2 min, 30 Hz) in a Tissue Lyser and Fibrous Tissue RNeasy Mini Kit, both from QIAGEN (Hilden, Germany). Three mice per condition were used for the gene expression profile analysis of GRK2$^{+/-}$ and WT mice of 4 and 9 months of age. cDNA synthesis, labeling and microarray analysis were performed with the aid of the Bioinformatics group at the National Center of Biotechnology (CNB, Madrid, Spain). Generation of double-stranded cDNA, preparation and labeling of cRNA, hybridization to GeneChip® Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) and washing were performed according to the protocol provided by Affymetrix. Probe sets were summarized using the LiMMA algorithm. Results were analyzed and visualized using Researcher's Digest software and FIESTA viewer respectively, as well as Multiexperiment Viewer software (TM4 Microarray Software Suite).

2.7. Co-immunoprecipitation assays

Cell lysates obtained as previously described for Western Blot were quantified using the Lowry procedure. Next, 500 μg total protein per lysate was incubated with 10 μl of either rabbit IgG or IRS1 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and 0.4 μg/μl BSA on a rotating shaker at 4 °C overnight and 15 μl of Protein G-Sepharose (50% in lysis buffer) were added to each tube. After 2 h on a rotating shaker at 4 °C, tubes were centrifuged at 14,000 ×*g* for 5 s and the pellets were washed with pre-chilled washing buffer (hypotonic-1% Triton) for 10 times (1 ml each). Pellets were then boiled in 4× Laemmli loading buffer for 5 min and used for Western Blot analysis. Blots were probed with specific antibodies against IRS1-PH [13] and GRK2 (Santa Cruz Biotechnology, Dallas, TX, USA).

2.8. RT-PCR and microarray validation

mRNA from heart tissue of at least 6 WT and 6 GRK2$^{+/-}$ mice was isolated as described before. RT-PCRs were performed by the Genomic Facility at CBMSO using Light Cycler equipment (Roche, Indianapolis, IN, USA). Microarray validations were performed using both commercial Taqman Gene Expression Assay probes (Applied Biosystems, Life Technologies, Grand Island, NY, USA) and self-designed probes purchased from Sigma with Syber Green technology. qPCRs and statistical analysis of the data were performed by the Genomic Facility at CBMSO using GenEx software.

2.9. Small-animal PET protocol

2-Deoxy-2-[18F]-fluoro-D-glucose in isotonic saline solution was injected through the intravenous catheter of WT and GRK2$^{+/-}$ adult mice to characterize heart glucose utilization in basal and after 5-min insulin stimulation. 90-minute dynamic imaging was performed with a piPET scanner and tomographic images were reconstructed using a three-dimensional ordered subset expectation maximization algorithm as previously described [15]. Region of interest measurements were made on multiple axial slices of the myocardium and tracer uptake was quantified as standardized uptake values normalized by injected dose and corrected for body weight.

2.10. Statistics

Data were analyzed by one-way ANOVA, followed by Bonferroni's post hoc analysis, or by unpaired T-testing when specified, as appropriate. For all tests, $p < 0.05$ was considered statistically significant after Bonferroni corrections, if needed, and all data are reported as means ± SEM.

3. Results

3.1. Decreased GRK2 levels correlate with enhanced cardiac insulin sensitivity

GRK2 can act as a negative modulator of insulin receptor signaling pathways, and adult GRK2$^{+/-}$ mice show improved systemic insulin sensitivity and are resistant to the induction of IR in peripheral tissues [2]. In this context, we explored the possibility that GRK2 dosage could modify heart insulin sensitivity in vivo in 9 month-old mice. The translocation to the plasma membrane of the GLUT4 glucose transporter is a key early event in glucose uptake stimulated by insulin. As can be observed in Fig. 1a, insulin-dependent GLUT4 translocation was significantly enhanced in adult GRK2 hemizygous mice, compared to wild-type animals, as assessed by Western Blot analysis of membrane fractions of cardiac tissue. These results are in agreement with the increased accumulation of labeled deoxyglucose in the hearts of GRK2$^{+/-}$ mice quantified by PET (Supplementary Fig. 1S). A similar pattern was observed for the rapid stimulation of Akt and its downstream target p70S6K kinase detected with specific phospho-antibodies upon injection of insulin (Fig. 1b–e).

Of note, upstream insulin signaling events such as the amount of phospho-Tyrosine(Tyr896)IRS1 were increased in GRK2$^{+/-}$ hearts after insulin stimulation while the activation status of the ERK cascade was not affected (Supplementary Fig. 1Sb–d). Together, these results demonstrate that the metabolic and pro-survival signals downstream of insulin were more potently activated in GRK2$^{+/-}$ hearts than in WT mice.

3.2. GRK2$^{+/-}$ 9 month-old mice display mild, non-pathological heart hypertrophy

The modulation of the PI3K/Akt and the ERK pathways in the heart is shared by insulin, IGF-1 and hypertrophic agonists such as angiotensin II. The PI3K/Akt cascade relates mostly to physiological hypertrophy, whereas MAPK signaling, together with PKC and calcineurin/NFAT, participates in the development of the pathological hypertrophy typically induced by angiotensin II (reviewed in ref. [16]).

We found that GRK2$^{+/-}$ mice showed a modest but significant increase in heart to body weight ratio and in the total cardiac area with age compared to the change observed in WT animals (Fig. 2a and b). We also found an enhanced increase in cardiomyocyte diameter in GRK2$^{+/-}$ animals (Fig. 2c), an established indicator of cardiac hypertrophy. No differences in these parameters were found at 4 months of age between WT and GRK2$^{+/-}$ animals. Echocardiographic analysis also revealed a certain degree of hypertrophy in the 9 month-old hemizygous mice, mainly referred to left ventricular mass (data not shown) in the absence of any alterations in cardiac functionality, as defined by fractional ejection (EF) or fractional shortening (FS) parameters (Fig. 2d). Consistently, fibrosis was not increased in either 4 or 9 month-old GRK2$^{+/-}$ mice compared with age-matched controls (Fig. 2e).

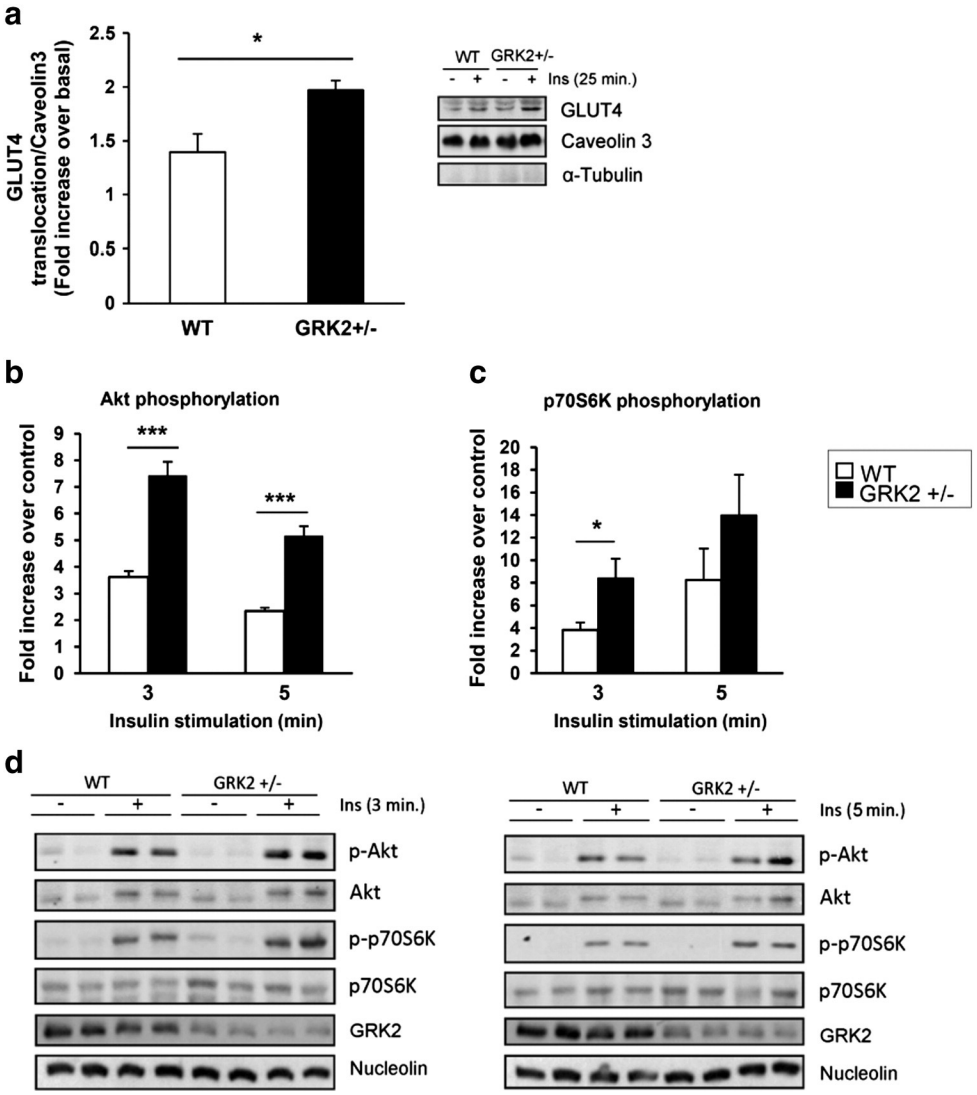


Fig. 1. GLUT4 translocation to the membrane and insulin signaling are upregulated in hearts from adult GRK2$^{+/-}$ mice. a). GLUT4 translocation in the plasma membrane fraction after an intravenous injection of insulin for 25 min in 9 month-old WT and GRK2$^{+/-}$ mice (N = 6–8 per genotype). Results were normalized to Caveolin 3 levels and are expressed as fold increase over basal (non-insulin treated mice). Representative blots are shown including α-Tubulin blot as an indicator of the absence of cytosolic contamination. Quantification of Akt phosphorylation (Ser473) (b) and p70S6K phosphorylation (Thr389) (c) in the cardiac tissue lysates of WT or GRK2$^{+/-}$ 9 month-old mice after an intravenous injection of insulin for 3 or 5 min (N = 3–5). Results are expressed as fold increase over control (non-insulin treated mice). d) Representative Western Blots of the specified phospho-proteins and controls in heart tissue 3 or 5 min after insulin injection. Data are mean ± SEM of the indicated independent experiments. ***$p < 0.001$; *$p < 0.05$.

3.3. Decreased GRK2 levels correlate with the expression of key genes involved in physiological hypertrophy/cardioprotection

Insulin is known to play a protective role in cardiac physiology, via the control of cardiac substrate utilization, cardiomyocyte growth, gene expression, survival and contractility by means of the homeostatic stimulation of the PI3K/Akt and other intracellular signaling pathways [17]. As an unbiased approach to assess the functional consequences of altering GRK2 levels in the modulation of insulin response and heart function, we compared the transcriptional profile of the cardiac tissue of WT and GRK2$^{+/-}$ mice of 4 or 9 months of age using microarray RNA expression techniques without subjecting the animals to prior specific treatments to study the integrated response of the tissue to homeostatic endogenous signals. Comparison of gene expression profiles between WT and GRK2$^{+/-}$ animals at each group of age (database access number GSE41706) revealed significant differences only at 9 months (33 genes significantly up-regulated and 28 genes down-regulated in GRK2$^{+/-}$ mice compared to WT) while no differences between genotypes were detected at 4 months of age (Fig. 3a and Supplementary Table 1), suggesting that the effects of GRK2 dosage on cardiac gene expression require additional age-related changes to become apparent.

We next performed a detailed analysis of the function and characteristics of the up or down-regulated genes. Several interesting patterns were noted. First, GRK2$^{+/-}$ mice at 9 months of age showed decreased expression of genes described to be up-regulated during pathological heart hypertrophy and/or in well-characterized cardiovascular disease co-morbidities such as diabetes and obesity. Eight out of the 28 genes down-regulated in GRK2$^{+/-}$ mice (29%) belonged to this group (see Fig. 3b). This list included the pivotal heart hypertrophy marker *Acta1* (skeletal muscle alpha-actin) and the atrial natriuretic factor precursor *Nppa* [18,19]; the cardiovascular heat shock protein *Hspb7*, with high expression in ob/ob mice skeletal muscle [20] and polymorphisms associated with development of idiopathic dilated cardiomyopathy and heart failure [21]; the transcription factor promyelocytic zinc finger protein (*Plzf*, also known as *Zbtb16*), a mediator of angiotensin 2-type 2 receptor-triggered cardiac hypertrophy [22]; the microfibril-associated glycoprotein-2 (*Magp-2*, also known as *Mfap5*), an angiogenic stimulator upregulated in transgenic models of HF [23], or the tissue inhibitor

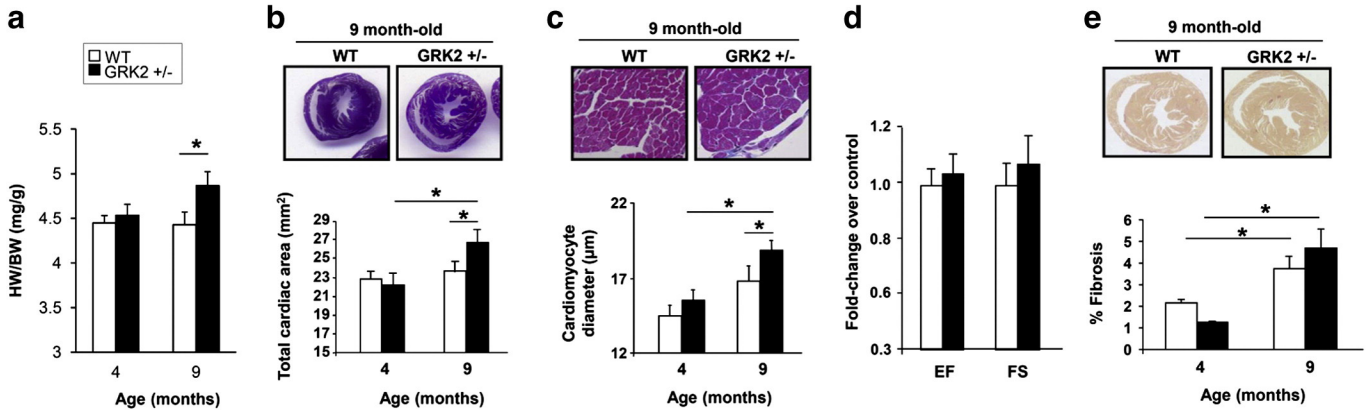
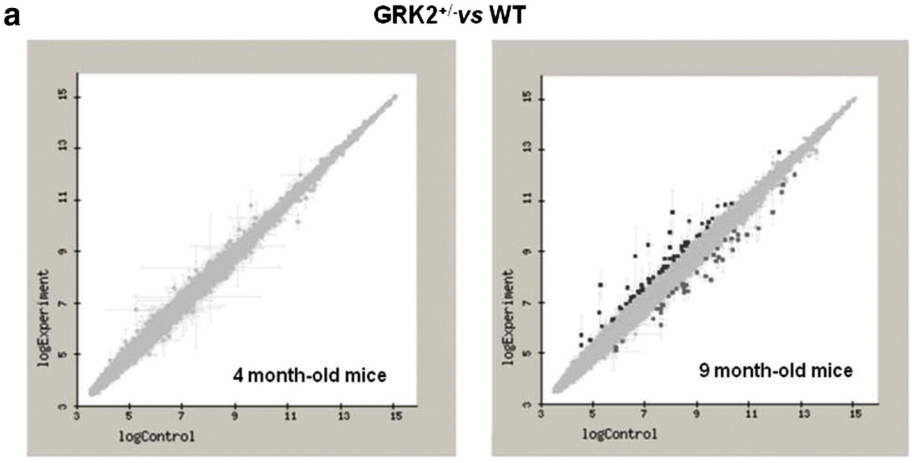


Fig. 2. GRK2$^{+/-}$ 9 month-old mice show mild, non-pathological heart hypertrophy. a) Heart weight (mg) to body weight (g) ratio in 4 and 9 month-old WT and GRK2$^{+/-}$ mice (N = 7–14). b) Total area of cross sections of each heart was measured from high-resolution images using the AnalySIS® software and expressed in mm^2 for WT (white bars) and GRK2$^{+/-}$ (black bars) mice (N = 5–9). c) Cardiomyocyte diameter (in μm) was measured in the cross-section of cells perpendicular to the slices stained with Masson's Trichrome in at least 20 cells per sample using the AnalySIS® software (N = 5–9). d) Fold change of ejection fraction (EF) and fractional shortening (FS), with statistical analysis performed using T-test. e) Quantification of the fibrotic area of heart cross sections stained with Syrius red, digitalized using the Axiovision software and analyzed using the AnalySIS® software. Representative pictures are shown when applicable. Data are mean ± SEM of the indicated independent experiments. *$p < 0.05$.



b

Genes	GRK2 +/- vs WT	Pathological heart hypertrophy Diabetes/Obesity
Rrad *Thbs1* *Pde4b* *Hdac9* *Nr4a1* *Tcf7L2* *Slc2a3* *Aqp7* *Meox2* *Ppargc1b*	Upregulated	Downregulated
Zbtb16 *Timp4* *Ptgds* *Acta1* *Nppa* *Hspb7* *Mfap5* *Pi3kr1*	Downregulated	Upregulated

Genes	GRK2 +/- vs WT	Physiological heart hypertrophy Response to exercise
Ppargc1b *Nr4a1* *Fosb* *Apold1*	Upregulated	Upregulated
Cebpb	Downregulated	Downregulated

Fig. 3. Comparison of cardiac gene expression profiles between young (4 month-old) or adult (9 month-old) WT and GRK2$^{+/-}$ mice. a) Genes whose expression varied significantly between WT and GRK2$^{+/-}$ mice are represented. Pairwise analyses were performed and visualized using the FIESTA viewer for an FDR < 0.2 (fold change > 1.5 or ≤1.5). No genes were found to change significantly at 4 months of age, but, at 9 months of age, 61 genes were significantly different between WT and GRK2$^{+/-}$ mice. Black, genes whose expression is increased in GRK2$^{+/-}$ vs WT. Gray, genes whose expression is reduced in GRK2$^{+/-}$ vs WT. b) The table depicts genes reported to be upregulated during pathological heart hypertrophy or during co-morbidities such as diabetes/obesity (see references in the text) found to be downregulated in adult GRK2$^{+/-}$ mice vs WT heart microarray, or genes for which the inverse relationship occurs (left panel). Also, a list of genes upregulated/downregulated in the array and also during physiological heart hypertrophy or response to exercise is specified (right panel).

of metalloproteinase 4 (*Timp4*), a proposed marker for left ventricular remodeling and deteriorating HF [24]. Prostaglandin D synthase (*Ptgds*), increased in the coronary circulation of angina patients [25] and overexpressed in type 2 diabetes [26] and the p85 alpha PI3K regulatory subunit (*Pik3R1*), enhanced in the myocardium of mice developing diet-induced obesity [27] and a key negative regulator of insulin signaling [28,29] were also decreased in 9 month-old GRK2$^{+/-}$ hearts.

A second interesting pattern found in GRK2$^{+/-}$ mice was that 10 out of 33 genes that were up-regulated in these animals (30%) have been reported to play a protective role in cardiovascular disease and are often down-regulated in pathological heart hypertrophy and/or diabetes/obesity co-morbidities (Fig. 3b). In this regard, the expression of *Ppargc1b*, *Hdac9*, *RRad*, or *Pde4b*, reported as key negative regulators of pathological heart hypertrophy, was enhanced in GRK2$^{+/-}$ mice. PGC1beta (peroxisome proliferator-activated receptor (PPAR)-gamma1beta co-activator) is essential for mitochondrial biogenesis and energy homeostasis. Decreased *Ppargc1b* expression correlates with cardiac insufficiency and cardiomyopathy and with obesity and type-2 diabetes, whereas its genetic deletion accelerates the transition to HF following pressure overload hypertrophy [30,31]. HDAC9 is an inhibitor of pathological (but not physiological) cardiac hypertrophy and *Hdac9*-deficient mice exhibit stress-dependent cardiomegaly [32]. RRAD (Ras associated with diabetes GTPase) levels are decreased in human failing hearts and in animal and cellular models of cardiac hypertrophy, and *RRad*-deficient mice are more susceptible to this condition [33]. RRAD appears to prevent CAMK-II-dependent hypertrophy, to inhibit cardiac fibrosis and to modulate beta-adrenergic mediated contractility [33,34]. Phosphodiesterase 4B (PDE4B) is an important, protective negative modulator of beta-adrenergic-mediated contractility with decreased levels in cardiac hypertrophy [35]. Thrombospondin 1 (*Thbs1*), a protein that modulates extracellular matrix metabolism and fibroblast phenotype, suggested to act as a protective signal in the stressed heart [36] was also enhanced in GRK2$^{+/-}$ mice, as was the anti-angiogenic homeobox gene *Meox2* [37].

The third relevant pattern of differential expression detected in GRK2$^{+/-}$ mice reflects changes in the expression of genes similar to those taking place upon exercise and in situations of physiological heart hypertrophy (Fig. 3b). These include the up-regulation of the central protective factors *Ppargc1b* and *Nr4a1* (see above); the early response genes *FosB* and *Apold 1*, reported to be enhanced in cardiac tissue upon acute physical activity [38] and the downregulation of the transcription factor C/EBPbeta (*Cebpb*), a member of the bHLH family. Importantly, the latter has been recently identified as a master regulator of physiological cardiac hypertrophy [39,40]. The mRNA expression of C/EBPbeta is reduced circa 60% (in the same range of our data) in mouse hearts in an exercise model allowing expression of an adaptive gene profile related to physiological hypertrophy, and mice with reduced cardiac levels of this protein displayed resistance to cardiac failure upon pressure overload [39]. The expression profile of several of the key genes supporting these patterns was validated by qPCR (Supplementary Fig. 2S).

In sum, the microarray analysis detected in GRK2$^{+/-}$ mice hearts an increased expression of a limited group of important genes related to physiological hypertrophy while the expression of genes key to the development of pathological hypertrophy or related to diabetes/obesity co-morbidities is repressed.

3.4. Increased cardiac GRK2 expression in adult obese (ob/ob) mice and in high fat diet-fed animals

Since GRK2 levels increase in muscle and adipose tissue under insulin resistance-promoting conditions [2] we tested whether this process was also taking place in cardiac tissue. A clear increase in GRK2 protein levels was observed in hearts of 8 month-old *ob/ob* mice (Fig. 4a), an age in which this strain of mice is known to manifest cardiac hypertrophy, IR and metabolic alterations involving alterations in the PI3K/Akt axis [41]. This increase in total GRK2 protein levels caused an increment in the amount of GRK2 that could be detected in association with IRS1 (Fig. 4b), a situation that we have previously described to negatively modulate insulin signaling. GRK2 levels were also increased upon feeding young animals a high fat diet (HFD, Fig. 4c), a well-established trigger for systemic IR that can promote cardiac remodeling and dysfunction and known to disrupt the insulin-stimulated IRS1/PI3K/Akt cascade [42]. This increase is similar to that observed in other insulin-sensitive tissues [2], and also had as a consequence a larger amount of formation of IRS1–GRK2 complexes (Fig. 4d). We investigated insulin responses in cardiac tissue of WT and GRK2$^{+/-}$ HFD-fed animals. Fig. 4e–f shows that sensitivity to insulin is compromised in WT hearts after HFD feeding, while it is strongly preserved in GRK2$^{+/-}$ hearts, in the absence of changes in insulin-induced ERK phosphorylation. Together these results suggest that the increase in GRK2 protein levels observed upon HFD feeding or genetically-induced obesity promotes GRK2-dependent sequestration of IRS1 and provides a mechanistic explanation for the IR state of cardiac tissue in both experimental conditions.

4. Discussion

We have addressed herein the potential interconnections among an IR status, GRK2 dosage and cardiac remodeling by investigating the impact of GRK2 down-modulation on cardiac insulin sensitivity and gene expression patterns, and the effects of systemic insulin resistance-promoting conditions on cardiac GRK2 levels.

First, we find that decreased GRK2 levels specifically preserve metabolic and pro-survival signals downstream of insulin (such as the Akt/p70S6K pathway and glucose transport) in the hearts of 9 month-old GRK2$^{+/-}$ animals, whereas the ERK activation status is not affected. Second, with age, GRK2 down-regulation triggers physiological heart hypertrophy and switches on a cardioprotective gene expression pattern as detected in such adult mice. This pattern is characterized by an increased expression of a limited group of key genes related to physiological hypertrophy, while the expression of genes reported to lead to the development of pathological hypertrophy or related to diabetes/obesity co-morbidities is repressed, compared to WT individuals. Third, we uncover that cardiac GRK2 expression levels increase in situations of systemic insulin resistance, such as in obese mice or after HFD feeding, conditions in which larger amounts of GRK2–IRS1 complexes are formed. Fourth, while insulin resistance develops in the hearts of wild-type mice after HFD feeding, sensitivity to insulin was strongly preserved in GRK2$^{+/-}$ cardiac tissue.

We have previously reported that GRK2$^{+/-}$ mice are protected against HFD-induced obesity and systemic IR, and we cannot rule out that this global protection may contribute to the observed enhanced insulin signaling in the heart upon a HFD. However, given the enhanced interaction of GRK2 with IRS1 that we find in cardiac tissue of different obese mice models, our data point at an important role for this kinase in negatively regulating insulin signaling in the heart, coherent with what was previously published by Garcia-Guerra et al. [2] involving the sequestration of IRS1 protein in GRK2 complexes thus impairing downstream signaling from the insulin receptor.

Previous reports have determined the importance of insulin signaling in cardiac physiopathology [43]. In particular, results in cardiomyocyte-specific deletion of the insulin receptor (CIRKO mice) have revealed that insulin can control cardiac gene expression patterns since loss of its receptor promotes a genomic reprogramming [44]. Insulin signals also control the size of the heart and cardiomyocytes, and deletion of insulin receptor decreases cardiac size by 20–30% [45]. These results are in agreement with the phenotype we find in GRK2 hemizygous mice in which the increased sensitivity for insulin signals results in gene expression reprogramming and an increased cardiac size.

The fact that GRK2 downregulation potentiates the insulin-triggered PI3K/Akt pathway in the adult mice heart is then consistent with the

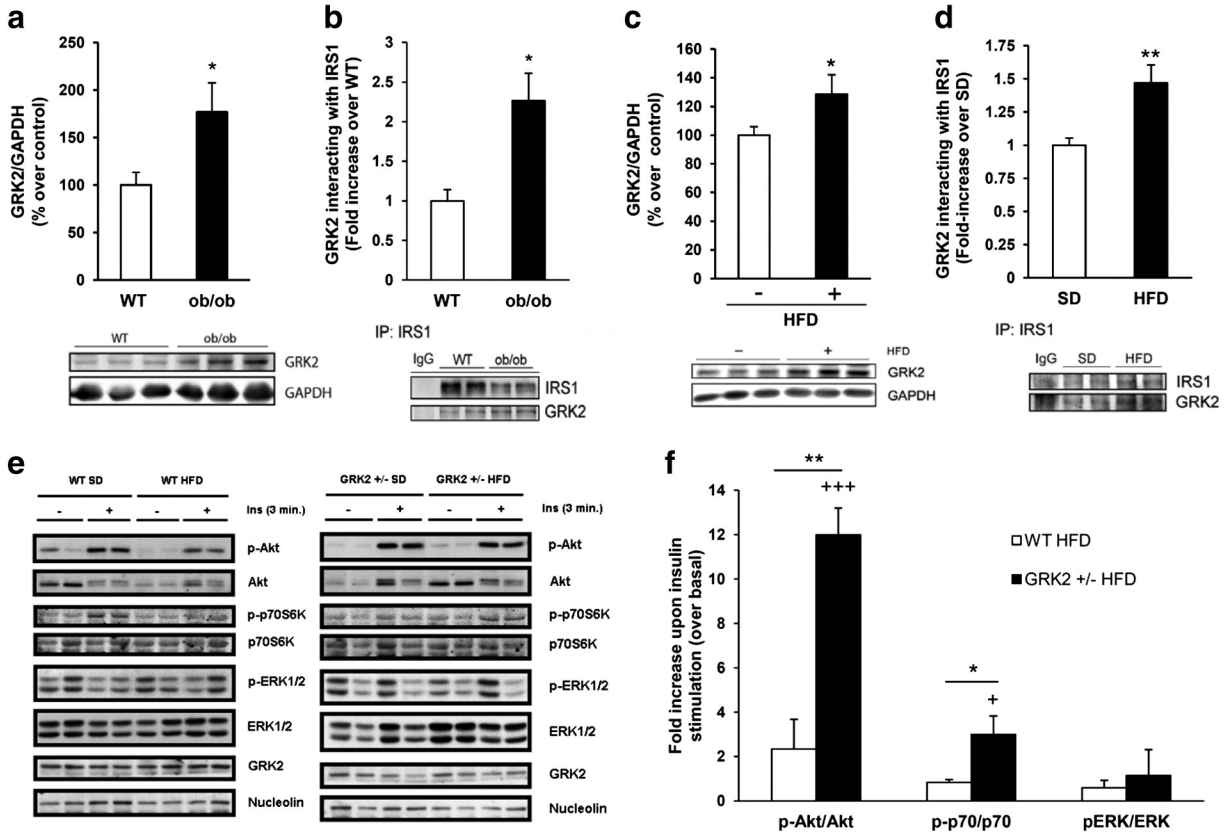


Fig. 4. Expression of the GRK2 protein is increased in myocardial tissue of obese or high fat diet-fed mice which correlates with higher levels of IRS1/GRK2 complexes, whereas GRK2$^{+/-}$ animals are resistant to high fat diet-induced insulin resistance in cardiac tissue. a) The expression levels of GRK2 were analyzed by Western Blot in cardiac tissue of 8 month-old ob/ob or WT mice and quantified by densitometry analysis. Results were normalized by GAPDH protein levels and expressed as percent over control (WT mice) (N = 5). b) Total protein from heart tissue (500 μg) was immunoprecipitated with the anti-IRS1 or anti-IgG antibodies, and the resulting immune complexes were analyzed by Western Blot with the corresponding antibodies against GRK2 and IRS1 (N = 3). Precipitated GRK2 amount was normalized with immunoprecipitated IRS1 amount. Same for 3 month-old HFD-fed mice represented over standard diet-fed animals in (c) and (d) (N = 9). Representative blots are shown. Data are mean ± SEM of the indicated independent experiments. **p < 0.01; *p < 0.05. e) Representative Western Blots of the specified phospho-proteins and controls in heart tissue after 12 weeks of HFD feeding or SD and 3 min insulin injection in WT or GRK2$^{+/-}$ mice. f) Quantification of Akt phosphorylation (Ser473), p70S6K phosphorylation (Thr389) or ERK1/2 phosphorylation (Thr202/Tyr204) in cardiac tissue lysates of WT and GRK2$^{+/-}$ mice after 12 weeks of HFD feeding after an intravenous injection of insulin for 3 min (N = 3–4). Results are expressed as fold increase over basal (untreated mice). Statistical analysis was performed using T-test. Data are mean ± SEM of the indicated independent experiments. +++p < 0.001; +p < 0.05 referred to basal (untreated mice); **p < 0.01; *p < 0.05 referred to fold increase upon insulin stimulation over HFD fed WT mice.

mild hypertrophic phenotype conferred by age to GRK2$^{+/-}$ animals being physiological rather than pathological, This is in agreement with the morphometric analysis and functional results, and with an overall cardio-protective gene expression pattern (decreased presence of pathological genes, enhanced expression of critical protective genes) in the heart of GRK2$^{+/-}$ 9 month-old mice. It is worth noting that GRK2$^{+/-}$ animals displayed changes in the expression of several genes reported to be similarly modulated by insulin in the heart or other tissues (*Nr4a1*, *Tcfl2*, *Rrad*, *Aqp7*, *Cebpb*, *Ppargc1b*, *Egr1*), and a relevant proportion of genes whose expression is altered in hemizygous animals can be related to: i) the modulation of insulin sensitivity; ii) the PI3K/Akt pathway; and iii) diabetes/obesity-related pathological situations.

Insulin activation of the PI3K/Akt cascade is protective in the heart by inhibiting apoptosis and oxidative stress [46], whereas myocardial IR has been suggested as a key factor in the development of HF [47,48]. In this context, our results strongly suggest the novel concept that cardiac GRK2 levels could act as an integrative sensor of different pathological inputs and affect cardiac function by simultaneously altering beta-adrenergic and insulin signaling (see suggested model in Fig. 5). Thus, the increase in cardiac GRK2 levels previously reported to take place in myocardial infarction or hypertension as a consequence of excessive neurohormonal stimulation [5,6], would also take place as a consequence of insulin resistance-promoting conditions such as a HFD or obesity, by mechanisms that remain to be investigated. Also, enhanced cardiac GRK2 would promote, in addition to the canonical effects described for GPCR signaling, an insulin-resistant state of the heart leading to alterations of key metabolic and cardioprotective pathways [11], further fuelling a dysfunctional cycle and allowing progression to maladaptive remodeling.

A detrimental vicious cycle has been postulated [48,49] in which the compensatory hyper-adrenergic state characteristic of reduced cardiac output would promote lipolysis in the adipose tissue. This would lead to increased circulating levels of free fatty acids, which in turn would inhibit cardiac glucose transport, switching energy substrate use and triggering heart lipotoxicity. Conversely, many mechanisms have been suggested to explain the increased incidence of HF in diabetic patients, including hyperinsulinemia, hyperglycemia, lipotoxicity, obesity, vascular alterations, increased oxidative stress or hyperactivation of neurohumoral systems ([47,48,50] and the references therein). Our data suggest that GRK2 could participate as an important integrative node in such complex mechanisms linking HF, diabetes and IR.

Such central role of GRK2 also fosters its potential as a therapeutic target and diagnostic marker. Our study suggests that strategies leading to a systemic reduction in GRK2 levels/function, even when used in a sustained temporal frame and in adult tissue, could facilitate the activation of defined cardioprotective routes, such as the insulin pathway. This would promote a physiological hypertrophy-like gene expression pattern that could contribute to explain the beneficial outcome of GRK2 inhibition.

This discovery could help explain the reported reinforcement between the therapeutic effects of down-regulating cardiac adrenergic input (using beta-blockers) and GRK2 down-modulation.

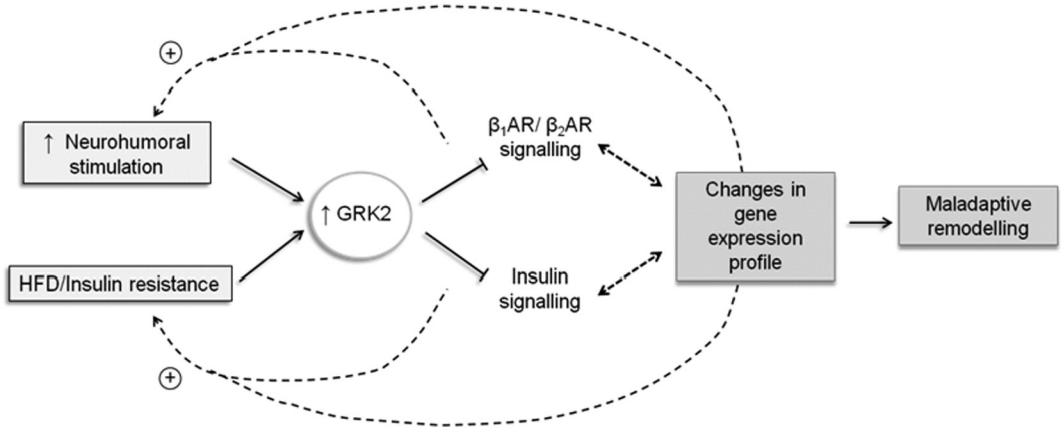


Fig. 5. Schematic representation of the proposed model of GRK2 up-regulation linking insulin-resistance and maladaptive cardiac remodeling. Increased GRK2 as a result of different pathophysiological situations would lead to a decrease not only in β-AR responsiveness, but also in insulin signaling thus promoting a pathological increase in the neurohumoral stimulation of the heart, and also lead to IR thus aggravating this condition. GRK2-promoted transcription reprogramming would also impinge upon both processes.

Since our data and previous report by other laboratories [1,6,7] indicate that reduced GRK2 revels are beneficial for cardiac function and also for maintaining vascular tone [4], and systemic insulin sensitivity [2], it could be argued that GRK2 hemizygosity would confer an overall benefit. However, the fact that a GRK2$^{+/+}$ genotype has been positively selected for by evolution suggests that the expression level generated by such genotype must be overall adaptative. Nevertheless, unlimited food availability, a condition that humans nowadays share with caged animals, was not at all present during evolution and therefore this might explain why GRK2 downregulation was not selected for.

GRK2 inhibition has been shown, in mouse models, to delay the reduction in glucose uptake and preserve insulin signaling in the heart after myocardial ischemia [11] and to prevent the development of systemic IR [2]. It is also worth noting that, apart from its catalytic kinase activity (target for potential GRK2 inhibitors), GRK2 plays an important functional role via protein–protein interactions. Our gene expression data cannot dissect which biological function of GRK2 needs to be reduced for therapeutic purposes, and it could well be possible that inhibition of the enzymatic activity of GRK2 might not reproduce the beneficial effects observed upon its under-expression. Thus, a better knowledge of means to reduce GRK2 levels in vivo, such as increasing its degradation rate or decreasing transcription, should be built before an effective GRK2 downmodulation therapy can be designed. Interestingly, ventricular assist device implantation, known to downregulate GRK2 levels [51], also reverses IR and normalizes cardiac metabolism in patients with advanced HF [52]. This is consistent with a beneficial role for GRK2 inhibition in this context. Notably, the reduction in GRK2 protein levels observed in lymphocytes of HF patients after an exercise-training program can predict long-term survival [53]. On the other hand, enhanced GRK2 levels in peripheral lymphocytes mimic myocardial levels during hypertension, myocardial ischemia and heart failure [6] and are also increased in patients with metabolic syndrome [2]. Therefore, it will be interesting to explore the potential use of GRK2 as a prognostic cardiovascular risk marker when co-morbidities such as diabetes, IR and obesity are present.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.09.004.

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Downregulation of G protein-coupled receptor kinase-2 levels enhances cardiac insulin sensitivity and switches on cardioprotective gene expression patterns

Elisa Lucas[1,2,&], María Jurado-Pueyo[1,2,&], María A. Fortuño[3], Sonia Fernández-Veledo[4], Rocío Vila-Bedmar[1,2], Luis J. Jiménez-Borreguero[2,5], Juan J. Lazcano[5], Ehre Gao[6], Javier Gómez-Ambrosi[7], Gema Frühbeck[7], Walter J. Koch[6], Javier Díez[3,8], Federico Mayor Jr.[1,2,*] and Cristina Murga[1,2,*].

[1] Departamento de Biología Molecular and Centro de Biología Molecular Severo Ochoa (UAM-CSIC), Madrid, Spain.

[2] Instituto de Investigación Sanitaria La Princesa, Madrid, Spain.

[3]Division of Cardiovascular Sciences, Centre for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain.

[4] Hospital Universitari de Tarragona Joan XXIII, IISPV, Universitat Rovira i Virgili, CIBERDEM Spain.

[5]Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain

[6] Department of Pharmacology and Center for Translational Medicine, Temple University, Philadelphia, USA.

[7] Metabolic Research Laboratory, Universidad de Navarra, CIBERobn,Pamplona, Spain.

[8]Department of Cardiology and Cardiovascular Surgery, University Clinic, University of Navarra, Pamplona, Spain.

[&]equal contribution to this work

[*] corresponding authors

SUPPLEMENTAL TABLES AND FIGURES

Supplemental Table 1S. Detailed list of genes showing differential expression between WT and GRK2$^{+/-}$ adult hearts.

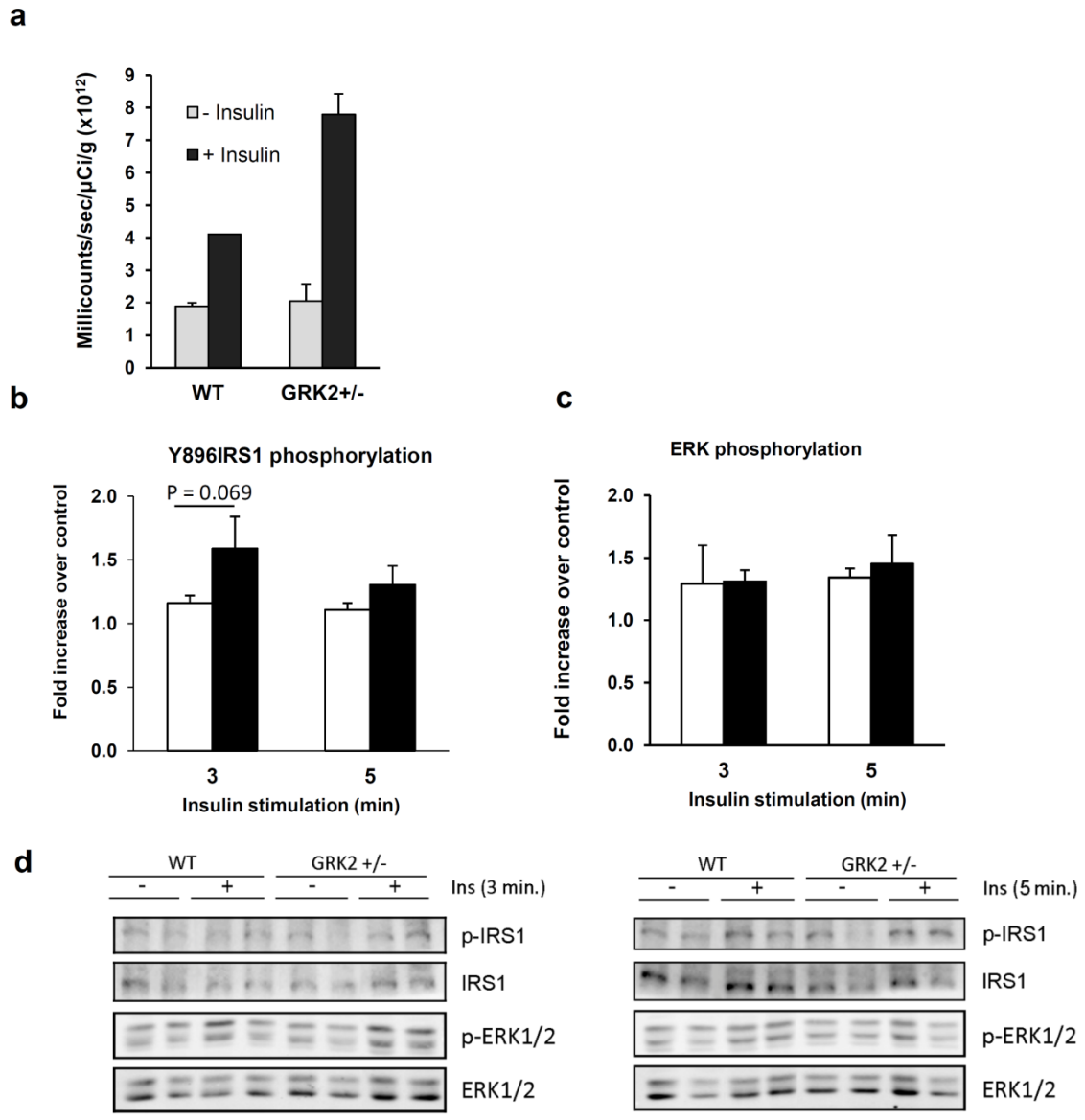
Genes whose expression varied with an FDR<0.2 and a Fold change >1.5 or <-1.5 are listed.

9 month-old +/- vs. 9 month-old wt

Fold-change	p-val (LiMMA)	Probe ID	Gene symbol/Gene title
+5.68	0.00087794	1417065_at	Egr1 | early growth response 1
+5.40	0.00001080	1436329_at	Egr3 | early growth response 3
+4.49	0.00001003	1448756_at	S100a9 | S100 calcium binding protein A9 (calgranulin B)
+3.59	0.00002794	1419394_s_at	S100a8 | S100 calcium binding protein A8 (calgranulin A)
+2.81	0.00002836	1416505_at	Nr4a1 | nuclear receptor subfamily 4, group A, member 1
+1.78	0.00031535	1435207_at	Dixdc1 | DIX domain containing 1
+1.73	0.00010339	1426952_at	Arhgap18 | Rho GTPase activating protein 18
+1.73	0.00000815	1449945_at	Ppargc1b | peroxisome proliferative activated receptor, gamma, coactivator 1 beta
+1.70	0.00097468	1421811_at	Thbs1 | thrombospondin 1 /// similar to thrombospondin 1
+1.69	0.00112735	1450407_a_at	Anp32a | acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
+1.66	0.00011584	1424234_s_at	Meox2 | mesenchyme homeobox 2
+1.66	0.00076817	1457644_s_at	Cxcl1 | chemokine (C-X-C motif) ligand 1
+1.64	0.00189368	1419156_at	Sox4 | SRY-box containing gene 4 /// similar to Transcription factor SOX-4
+1.64	0.00029988	1447457_at	Ada | Adenosine deaminase
+1.61	0.00039396	1418849_x_at	Aqp7 | aquaporin 7
+1.59	0.00035215	1436600_at	Tnrc9 | trinucleotide repeat containing 9
+1.58	0.00023340	1437052_s_at	Slc2a3 | solute carrier family 2 (facilitated glucose transporter), member 3
+1.58	0.00026865	1439766_x_at	Vegfc | vascular endothelial growth factor C
+1.58	0.00242113	1456735_x_at	Acpl2 | acid phosphatase-like 2
+1.57	0.00001115	1433651_at	Wtip | WT1-interacting protein
+1.56	0.00005950	1457432_at	Prox1 | prospero-related homeobox 1
+1.55	0.00088244	1422134_at	Fosb | FBJ osteosarcoma oncogene B
+1.55	0.00001032	1422474_at	Pde4b | phosphodiesterase 4B, cAMP specific
+1.54	0.00181760	1434856_at	Ankrd44 | ankyrin repeat domain 44
+1.53	0.00129704	1448382_at	Ehhadh | enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
+1.53	0.00020216	1457139_at	Auts2 | autism susceptibility candidate 2
+1.52	0.00007822	1422562_at	Rrad | Ras-related associated with diabetes
+1.52	0.00203143	1423489_at	Mmd | monocyte to macrophage differentiation-associated
+1.52	0.00034652	1441228_at	Apold1 | apolipoprotein L domain containing 1
+1.52	0.00018939	1444952_a_at	Nucks1 | nuclear casein kinase and cyclin-dependent kinase substrate 1
+1.51	0.00011940	1429427_s_at	Tcf7l2 | transcription factor 7-like 2, T-cell specific, HMG-box
+1.51	0.00050197	1434572_at	Hdac9 | histone deacetylase 9
+1.51	0.00168651	1456005_a_at	Bcl2l11 | BCL2-like 11 (apoptosis facilitator)

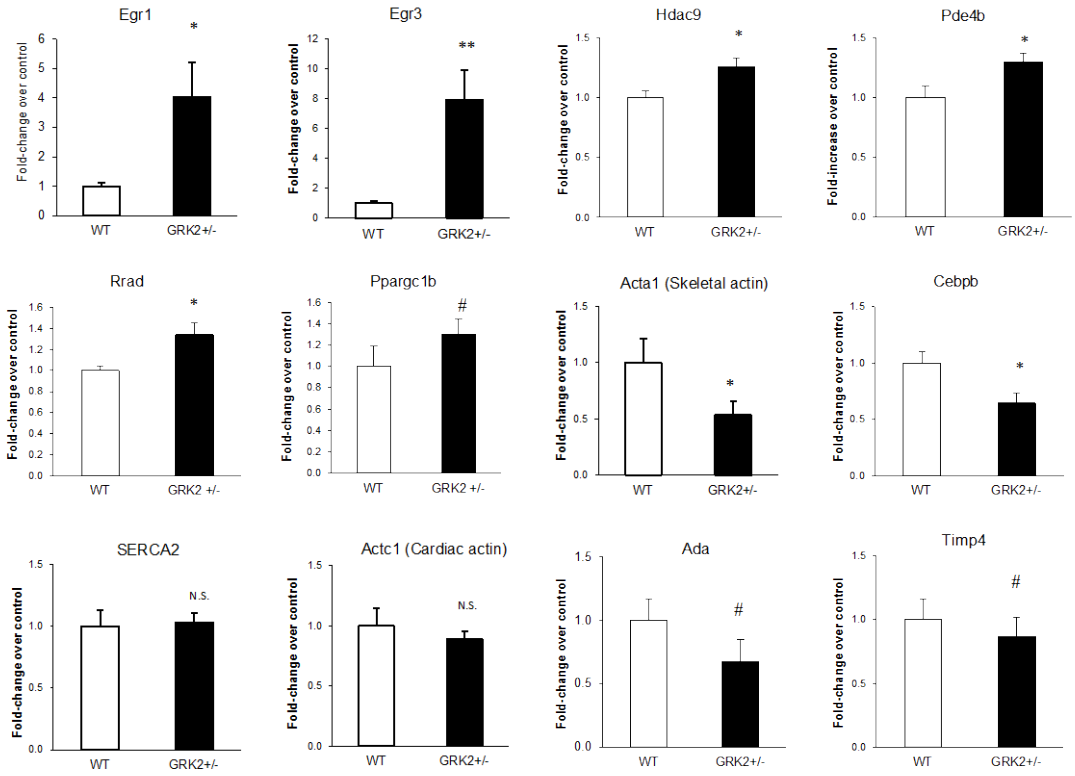
Fold-change	p-val (LiMMA)	Probe ID	Gene symbol/Gene title
-1.51	0.00052884	1423233_at	Cebpd | CCAAT/enhancer binding protein (C/EBP), delta
-1.51	0.00128321	1425281_a_at	Tsc22d3 | TSC22 domain family 3
-1.53	0.00079516	1419598_at	Ms4a6d | membrane-spanning 4-domains, subfamily A, member 6D
-1.53	0.00190063	1435459_at	Fmo2 | flavin containing monooxygenase 2
-1.54	0.00052036	1448185_at	Herpud1 | homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
-1.54	0.00016858	1449082_at	Mfap5 | microfibrillar associated protein 5
-1.56	0.00029120	1428547_at	Nt5e | 5 nucleotidase, ecto
-1.57	0.00216211	1421289_at	Hspb7 | heat shock protein family, member 7 (cardiovascular)
-1.58	0.00169451	1425515_at	Pik3r1 | phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)
-1.58	0.00088349	1455399_at	Cnksr1 | connector enhancer of kinase suppressor of Ras 1 /// similar to connector enhancer of kinase suppressor of Ras1
-1.60	0.00025712	1454638_a_at	Pah | phenylalanine hydroxylase
-1.67	0.00001101	1428776_at	Slc10a6 | solute carrier family 10 (sodium/bile acid cotransporter family), member 6
-1.68	0.00055243	1438009_at	MGC73635 | similar to histone 2a
-1.72	0.00063349	1434437_x_at	Rrm2 | ribonucleotide reductase M2
-1.72	0.00004522	1439153_at	Ibrdc2 | IBR domain containing 2
-1.73	0.00011780	1427844_a_at	Cebpb | CCAAT/enhancer binding protein (C/EBP), beta
-1.78	0.00214243	1425645_s_at	Cyp2b10 | cytochrome P450, family 2, subfamily b, polypeptide 10
-1.81	0.00008762	1427638_at	Zbtb16 | zinc finger and BTB domain containing 16
-1.85	0.00010205	1427345_a_at	Sult1a1 | sulfotransferase family 1A, phenol-preferring, member 1
-1.89	0.00012300	1425303_at	Gck | glucokinase
-1.90	0.00256270	1423860_at	Ptgds | prostaglandin D2 synthase (brain)
-1.94	0.00108646	1428352_at	Arrdc2 | arrestin domain containing 2
-2.16	0.00058602	1450974_at	Timp4 | tissue inhibitor of metalloproteinase 4
-2.32	0.00063394	1456062_at	Nppa | natriuretic peptide precursor type A
-2.39	0.00002699	1416125_at	Fkbp5 | FK506 binding protein 5
-2.43	0.00002224	1416225_at	Adh1 | alcohol dehydrogenase 1 (class I)
-2.47	0.00164694	1427735_a_at	Acta1 | actin, alpha 1, skeletal muscle
-2.76	0.00070986	1417600_at	Slc15a2 | solute carrier family 15 (H+/peptide transporter), member 2

Supplemental Figure 1S



Suppl. Fig.1S.- Glucose uptake and insulin signalling are upregulated in hearts from adult GRK2+/- mice without differences in pERK activation. a) Positron emission tomography (PET) analysis of insulin-dependent 18F-Fluoro deoxy-glucose uptake in the hearts of 9 month-old WT and GRK2+/- mice (N=3 per genotype). Quantification of IRS1 phosphorylation (Tyr896) (b) and ERK1/2 phosphorylation (Thr202/Tyr204) (c) in the cardiac tissue lysates of WT or GRK2+/- 9 month-old mice after an intravenous injection of insulin for 3 or 5 minutes (N=3-5). Results are expressed as fold increase over control (non-insulin treated mice). d) Representative Western Blots of the specified phospho-proteins and controls in heart tissue 3 or 5 min after insulin injection. Data are mean±SEM of the indicated independent experiments.

Supplemental Figure 2S



Suppl. Fig.2S.- Validation by qRT-PCR analysis**.** A qRT-PCR analysis was performed for certain genes whose changes in expression are described to correlate with physiological or pathological cardiac hypertrophy or other cardiac responses important for cardiac physiopathology as discussed in the main text. * $p<0.05$, # $0.2>p>0.05$.

INTRODUCTION TO ARTICLE #4

The fourth article presented is entitled Obesity-related cardiac hypertrophy is modulated by G protein-coupled receptor kinase 2. (In preparation for submission)

The main objective of this work was to determine whether low levels of GRK2 would protect from obesity-induced cardiac remodeling. For this purpose we fed mice with HFD for 33 weeks (animals were 10 month-old at the end of the study), followed by analysis of the cardiac tissue once long-term obesity has been established. We concluded that a partial deficiency of GRK2 promotes a leaner and insulin-sensitive systemic phenotype and preserves from cardiac ectopic fat accumulation and hypertrophy.

This could be the first report indicating a protective role of GRK2 downmodulation in obesity-induced cardiac remodeling, what could be of great importance to the field given the rising incidence of obesity in the population and its implications in cardiovascular diseases.

As first author of this manuscript I have been involved in all aspects of this work and have been the principal responsible researcher for all the experimental work and design.

Obesity-related cardiac hypertrophy is modulated by G protein-coupled receptor kinase 2

Lucas E.[1,2], Vila-Bedmar R.[1,2], Mayor Jr. F.[1,2] and Murga C.[1,2]

1 Departamento de Biología Molecular and Centro de Biología Molecular Severo Ochoa (UAM-CSIC), Madrid, Spain.
2 Instituto de Investigación Sanitaria La Princesa, Madrid, Spain.

ABSTRACT

Obesity is a growing problem that has become a public health and clinical challenge worldwide. The “obesity epidemic” is principally driven by an imbalance between energy intake and energy expenditure. Although obesity is related to type 2 diabetes and certain types of cancer, the leading cause of death among the obese population is heart failure and stroke. Moreover, obesity is strongly associated with the development of major risk factors for cardiovascular disease such as hypertension, dyslipidemia and diabetes promoting structural and functional changes in the heart. The molecular mechanisms that underlie obesity-related cardiac remodeling are complex, and include hemodynamic and metabolic alterations that ultimately impact the myocardium. G protein-coupled receptor kinase 2 (GRK2) is a ubiquitous serine/threonine protein kinase able to phosphorylate and desensitize the active form of several G protein-coupled receptors (GPCR) and known to play an important role in cardiac GPCR modulation. GRK2 has also been recently identified as a negative modulator of insulin signaling and systemic insulin resistance. In this context, we investigated the effects elicited by GRK2 down-regulation in obesity-related cardiac remodeling using adult 10 month-old mice fed with a high fat diet (HFD) for 33 weeks. We find that GRK2+/- mice, which display circa 50% lower levels of this kinase, are protected from obesity-promoted cardiac hypertrophy, as assessed by using both molecular markers and morphometrical measurements of cardiac tissue and cells. These mice are also resistant to the excessive intracellular lipid accumulation in cardiomyocytes (cardiac steatosis) triggered by HFD in control littermates. Interestingly, HFD significantly increased cardiac GRK2 levels in wild type but not in GRK2+/- mice, suggesting that the beneficial phenotype observed in hemizygous animals correlates with keeping GRK2 levels below a pathological threshold. Our data open new avenues of research to further define the cellular processes and molecular mechanisms by which GRK2 down-regulation is cardioprotective during diet-induced obesity

INTRODUCTION

The dramatic increase in the incidence of obesity and its strong association with cardiovascular disease and overall mortality has resulted in an unprecedented interest in understanding the effects of obesity on the cardiovascular system Obesity, or “Globesity”, as it has been referred by the World Health Organization given the ever-increasing prevalence of obesity world-wide, is a complex condition that affects virtually all age and socioeconomic groups and threatens to overwhelm both developed and developing countries. It has been estimated that by 2015, approximately 2.3 billion adults will be overweight and more than 700 million will be obese [1]

Although obesity is most commonly caused by a disruption in energy homeostasis due to the imbalance between dietary energy consumption (calorie-dense food and drinks) relative to energy expenditure (energy loss via metabolic and physical activity), the etiology of obesity is highly complex and includes genetic, physiologic, environmental, psychological, social and economic factors that interact in varying degrees to promote the increase in body fat mass and the development of obesity [2]. Besides an altered metabolic profile, a variety of adaptations/alterations in cardiac structure and function occur in the individual as adipose tissue accumulates in excessive amounts, even in the absence of comorbidities such as type 2 diabetes and hypertension [3]. For instance, the mass of the left ventricle has been shown to

grow and correlate proportionally with body weight [4]. Finally, prolonged existence of obesity causes both left ventricular systolic and diastolic functions [5].

In humans, increased cardiac mass has been postulated to result from increased epicardial fat and fatty infiltration of the myocardium [6]. Moreover triglyceride content in human myocardium was found significantly increased in obese compared with normal-weight subjects [7]. Increased accumulation of intramyocellular triglycerides in the heart is also a commonly described feature of most animal models of obesity [8-10] and the accumulation of myocardial triglyceride and lipid metabolites such as ceramides has been found to be lipotoxic as it has been associated with cardiomyocyte apoptosis [11].

Another common feature in the obese heart is impaired insulin signaling. It starts to develop within two weeks of high fat diet (HFD) [12] and therefore represents an early adaptation of the heart to caloric excess that promotes the development of diabetic cardiomyopathy. This condition not only alters cardiac metabolism but also increases myocardial oxygen consumption, reduces cardiac efficiency by uncoupling of the mitochondria and increases oxidative stress [13].

G protein-coupled receptor kinase 2 (GRK2) is a serine/threonine kinase originally discovered to regulate G protein-coupled receptor (GPCR) desensitization and known to play an important role in cardiac function and dysfunction and to display increased levels in cardiac hypertrophy and heart failure. In addition, GRK2 is emerging as an important signaling hub with a complex interactome [14, 15], and has recently been identified as a direct modulator of insulin signaling in several tissues, including the heart [16, 17]. Interestingly, GRK2+/- mice (expressing some 50% less protein than control littermates) show improved systemic insulin sensitivity in different mice models [17, 18]. On the other hand, we have recently described that GRK2 levels are increased in the hearts of adult ob/ob mice as well as in mice fed with a high-fat diet (HFD) for 12 weeks [17]. Given the emerging role of GRK2 as a regulatory hub in heart physiology we have explored the role of GRK2 dosage in the development of obesity-induced cardiac remodeling and steatosis in 10-month old mice, considering that is in adulthood when obesity-related cardiac pathological events become more apparent.

MATERIALS AND METHODS

Animals: Experiments were performed on male wild type (WT) and hemizygous-GRK2 (GRK2+/-) mice maintained on the hybrid 129/SvJ C57BL/6 background. The animals were bred and housed on a 12-hour light/dark cycle with free access to food and water. GRK2+/- mice and their corresponding wild types (C57BL/6J, The Jackson Laboratory, Bar Harbor, ME, USA) were fed ad libitum since weaning either on a normal chow diet or standard diet (SD, providing 13% of total calories as fat, 67% as carbohydrate and 20% as protein; 2014S Rodent Maintenance Diet, Teklad, Harlan, Barcelona, Spain) or a high fat diet (HFD, providing 45% of total calories as fat, 35% as carbohydrate and 20% as protein, Rodent Diet D12451, Research Diets, New Brunswick, NJ, USA) for 33 weeks. Animals were maintained at a room temperature of 22±2 ºC with a relative humidity of 50±10% and under pathogen-free conditions. Body weight and food intake were measured weekly. All animal experimentation procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals (Directive 86/609) and approved by the Ethical Committees for Animal Experimentation of the Universidad Autónoma de Madrid.

Metabolic assays: Insulin tolerance test (ITT) was performed as previously described [19]. Animals were fasted for 4 hours (10:00–14:00), and baseline blood samples were collected from the tail of fully conscious mice. Insulin (0.8 U/kg body weight) was administered by i.p. injection, and blood samples were taken from the tail at 15, 30, 45, and 60 minutes after injection. Glucose concentration (mg/dl) was determined in tail blood samples using an automatic analyzer (One Touch Ultra), from Life Scan.

Heart collection and processing: Mice were euthanized using CO_2 and weighted. Hearts were surgically removed and immediately weighted. Auricles were removed and ventricles were sliced transversally in four portions. The two central slices were fixed in 4%

paraformaldehyde and embedded in paraffin or Tissue-Tek® OCT for histological analysis. The other two portions were frozen in liquid nitrogen for protein and gene expression analysis.

Cardiomyocyte hypertrophy determination: Paraffin blocks of heart slices were cut 6 μm thick and stained with Masson´s trichrome for the evaluation of cardiomyocytes area. For each experimental group, four sections were stained per animal. Digital images of those areas in the heart ventricle where cardiomyocytes were transversally cut were captured using a light microscope (Olympus, Germany) at 20X magnification. For each condition four mice hearts were employed and three fields were analyzed per mouse. Individual cardiomyocyte areas within each field were determined using image analysis software (ImageJ). Relative cardiomyocyte size was calculated by quantification of 150-200 cells per field.

Gene expression analysis: mRNA from heart tissue of at least 6 mice per condition was isolated as described in [17]. RT-PCRs were performed by the Genomic Facility at CBMSO using Light Cycler equipment (Roche, Indianapolis, IN, USA). Gene expression quantifications were performed using both commercial Taqman Gene Expression Assay probes (Applied Biosystems, Life Technologies, Grand Island, NY, USA) and self-designed probes purchased from Sigma labeled with Syber Green. qPCRs and statistical analysis of the data were performed by the Genomic Facility of our institute using GenEx software.

Western Blot analysis: Heart tissue was homogenized as described in [17]. Tyipically 40 μg of total cardiac protein was resolved per lane on a 7.5% SDS-PAGE gel and transferred to a nitrocellulose membrane. Blots were probed with specific antibodies against GRK2 and nucleolin (Santa Cruz Biotechnology, Dallas, TX, USA).

Intracellular lipid droplet quantification: OCT frozen blocks of heart tissue were cut in 6 μm thick slices, mounted on 10% glycerol in PBS-DAPI (5 ng/μl) to visualize the nucleus, and stained with Oil red O as described (Koopman R et al, 2001). All sections were examined using a Fluorescence Resonance Energy Transfer (FRET) equipment coupled to an inverted Axiovert200 (Zeiss, Germany) microscope. Oil red O-stained sections were examined in epifluorescence using a DsRed (500–650 nm) and DAPI (359-371 nm) excitation filter. Digital images of arbitrary fields were captured at 100x magnification. For each condition three different hearts and 10 fields per mouse were analyzed. Total lipid droplet content per total cellular area was determined using image analysis software (ImageJ) which was also used for the characterization and quantification of thresholded droplet areas within each field.

Stadistical analysis: All data are expressed as mean values ± SEM and N represents the number of animals. Statistical significance was analyzed by using unpaired Student's t test or one-way ANOVA followed by Bonferroni's post hoc test. Differences were considered statistically significant when P value <0.05.

RESULTS

Decreased levels of GRK2 attenuates the diet-induced obesity phenotype

Two months after birth mice were fed either a standard diet (SD) or high fat diet (HFD) for 33 weeks. Both genotypes significantly gained weight after HFD feeding compared with SD-fed mice (Fig. S1), but GRK2+/- mice maintained a leaner phenotype compared with wild type (WT) in the absence of differences in food intake (Fig. 1a and b). Since systemic insulin resistance is a common comorbidity in obese individuals, we performed insulin tolerance tests (ITTs) in animals of each genotype. We observed that GRK2+/- adult mice were more sensitive to insulin than their WT littermates when fed a SD (Fig. 1c), in line with previously published results [18]. Interestingly, such enhanced insulin sensitivity detected upon GRK2 downregulation was even more significant after a long-term HFD feeding (Fig. 1d). Indeed, insulin tolerance in long-term HFD-fed GRK2+/- mice was comparable to that of SD-fed WT

mice as reflected by analyisis of the area under the curve (Fig. 1e). These results build on previous data from our laboratory that showed improved maintenance of body weight and insulin sensitivity in GRK2+/- mice after a 12-week HFD, indicating that these animals are able to preserve weight gain and maintain insulin sensitivity compared to WT littermates even after a long-term 30 week HFD feeding when they are already 10 month-old.

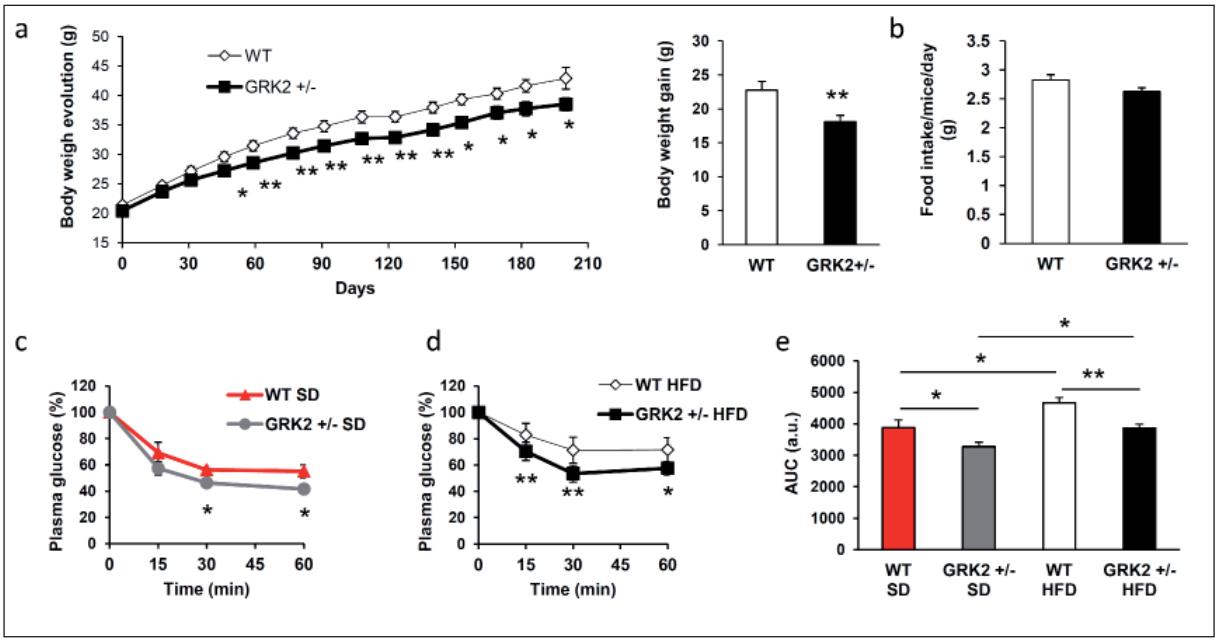


Figure 1: **GRK2+/- animals show an attenuated obese phenotype after long-term HFD feeding.** (a) Comparison of body weight evolution and body weight gain (expressed in g) between WT and GRK2+/- after 30 weeks of HFD feeding. (b) Daily food intake (expressed in g) (N=7). (c-d) Intraperitoneal insulin tolerance tests (ITT) in SD- and HFD-fed mice and (e) histogram showing the product of ITTs area under the curve (AUC) (N=6-10). Data are means ± SEM of the indicated independent experiments. * P<0.05, **P<0.01, ***P<0.001.

Lower levels of GRK2 protect hearts from high fat diet-induced hypertrophy

In a previous work [17] we reported that lower levels of GRK2 could protect the heart from 12 weeks HFD-induced insulin resistance. However, such short period of HFD feeding seemed not to be sufficient for the development of cardiac hypertrophy in WT mice (Fig. S2). So, in order to determine if obesity-induced cardiac remodeling could be affected by GRK2 dosage, we set out to study the heart morphometry in the longer-term model of 33 weeks on HFD. We first observed that HFD feeding provoked cardiomegaly in WT animals while HFD-fed GRK2+/- mice heart sizes were indistinguishable from those of SD-fed mice (Fig. 2a). Interestingly, a similar effect was also observed at a cellular level where a reduction in GRK2 prevented the increase in cell size that was clearly observed in WT mice after 33 weeks of HFD (Fig. 2b), even considering that hemizygous animals display a mild, non-pathological cardiomyocyte hypertrophy in SD conditions, in agreement with previous reports [17]. In sum, decreased levels of GRK2 abrogate obesity-induced cardiomyocyte and heart hypertrophy after a long-term HFD feeding in adult 10 month-old mice.

Higher levels of GRK2 protein correlate with the expression of pathological hypertrophy-related markers

In consistency with previous results [17] we found that a 33-week long HFD induced a increase of cardiac GRK2 levels, but this increase was only significant in WT mice, while GRK2+/- animals maintained lower levels of GRK2 after such long term HFD feeding (Fig 3a).

In order to characterize whether the obesity-induced cardiac hypertrophy that we detected was of a pathological nature, we quantified the mRNA expression levels of several established markers of this condition such as α-skeletal actin (Acta1), a prototypical marker of cardiac hypertrophy, sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA2), a marker of cardiac

functionality that is decreased in most cases of hypertrophied failing hearts, and B-type natriuretic peptide (BNP), a highly sensitive marker of cardiac pathology/stress. We included GRK2 mRNA in the analysis in order to study if the observed increase in GRK2 protein levels resulted from changes in the transcriptional control of this gene. The gene expression profile for Acta1 precisely correlates with the degree of cardiomyocyte hypertrophy observed thus confirming that HFD induced a marked cardiac hypertrophy in WT but not in GRK2+/- animals. We found no significant changes in SERCA2 expression in either genotype of feeding regime, but there is a tendency towards an increased expression of SERCA2 in SD-fed GRK2+/- mice compared to SD-fed WT animals that would indicate an amelioration of cardiac functionality upon GRK2 downregulation in this model, what is in line with previously reported results [17, 20]. Interestingly, GRK2+/- mice were also protected from the increase in BNP expression after long term HFD feeding. We detect no changes in GRK2 mRNA after HFD feeding (Fig. 3b) what suggests that the increase in cardiac GRK2 protein levels observed after HFD feeding probably responds to post-translational regulation. In sum, these data indicate that decreased GRK2 dosage would help to maintain a physiological gene expression profile in conditions of long-term HFD feeding leading to the development of cardiac hypertrophy in WT animals.

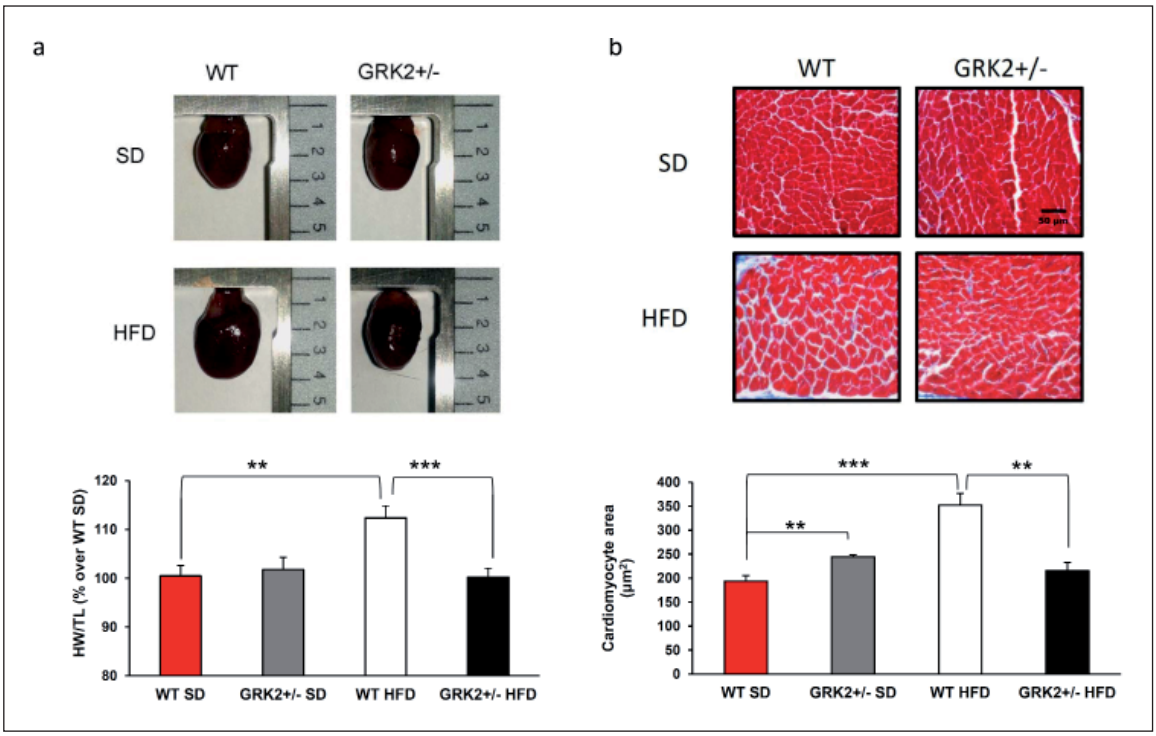


Figure 2: **Lower levels of GRK2 protect mice hearts from long-term (33 weeks) HFD-induced hypertrophy.** (a) Heart weight to tibial length ratio in HFD-fed 10 month-old WT and GRK2+/- mice, compared with their littermates fed with standard diet (N= 9). (b) Cardiomyocyte cross-sectional area from each genotype expressed in μm^2 (N = 6). Representative images are shown in 20x magnification. Scale bar, 50 μm. Data are means ± SEM of the indicated independent experiments. **P<0.01, ***P<0.001.

GRK2+/- mice are protected from excessive intra-myocardial lipid accumulation

One of the ultimate pathological features of hearts from obese individuals is the accumulation of lipid droplets in the cardiac cell that can promote lipotoxicity. Staining of fat depots with Oil red O (Fig. 4a) showed that the amount of intracellular lipid droplets was significantly lower in either HFD- or SD-fed GRK2+/- mice hearts than in their littermate counterparts. Indeed, the amount of intracellular fat was almost the same for GRK2+/- mice after SD or after HFD, whereas it significantly increased in WT cardiac cells after HFD feeding (Fig. 4b). Interestingly, the mean size of intracellular lipid depots was larger in HFD-fed WT cardiomyocytes than in any other condition (Fig. 4d). These results suggest that GRK2 downregulation regulates cadiac lipid storage and prevents the pathological accumulation of lipid inside cardiac cells after a long-term HFD feeding.

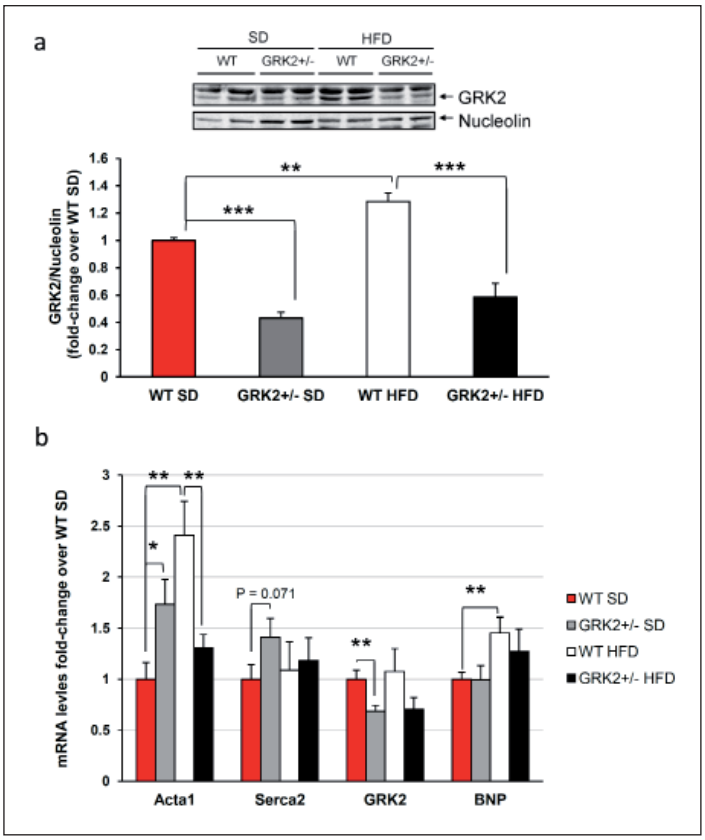


Figure 3: **Levels of GRK2 correlate with a hypertrophic marker profile in long-term HFD-fed animals.** (a) GRK2 protein levels in cardiac tissue normalized by Nucleolin levels are expressed as fold-increase over SD-fed WT animals (N=3-4). (b) Expression of several markers of heart hypertrophy (Acta1), cardiac functionality (Serca2) and cardiac stress (BNP) were quantified by qPCR. GRK2 was used as internal control (N=6). Data are means ± SEM of the indicated independent experiments. * P<0.05, **P<0.01, ***P<0.001.

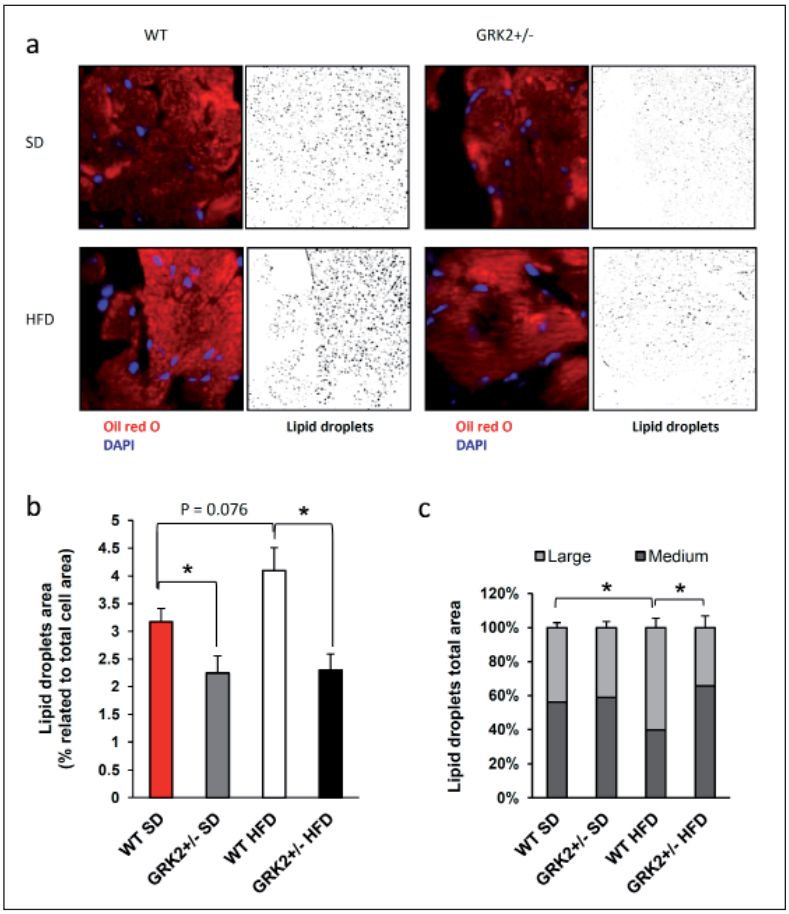


Figure 4: **Intracellular lipid accumulation is enhanced in WT compared with GRK2+/- littermates after standard or long-term HFD feeding.** (a) Frozen heart sections were stained with Oil red O and DAPI to visualize and quantify intracellular lipid droplets and nuclei, respectively. Lipid droplets in each condition are shown in black and white after image processing. Results are from at least 10 photomicrographs from each heart (magnification 100x) (N=3). (b) Percentage of the area occupied by lipid droplets in cardiomyocytes for each condition. (c) Lipid droplet´s area were classified according to their sizes as medium (0.5-1 μm^2) or large (>1 μm^2). Total area of lipid droplets of each size was normalized with total area of lipid droplets. Statistical analysis was performed using T-test. Data are mean±SEM of the indicated independent experiments. *p<0.05

DISCUSSION

In this work we present some preliminary lines of evidence that point to a protective role of lowering GRK2 levels upon obesity-induced cardiac remodeling. First, we find that adult GRK2+/- mice gained less weight than WT littermates after a long period (33 weeks) of HFD feeding. We have previously described [17, 18] that GRK2+/- mice display protection against 12 weeks of HFD-induced obesity. In the current model the mice have been fed for a longer time with HFD hence they reached adulthood (10 months-old) in such conditions, contrary to most of the studies where animals are fed with HFD for shorter time frames and thus analysis are performed when they are still young (typically 5-6 month old). We claim that our experimental approach would more reliably mimic the current profile of human obesity whose incidence in middle-age adults is higher than in the young population, as is the presence of associated comorbidities as cardiovascular disease [21]. Second, after a long-lasting HFD feeding period, the insulin sensitivity observed in GRK2+/- mice remains comparable to that observed in aged-matched SD-fed WT animals, indicating that a lower GRK2 dosage protects against the diet-induced insulin resistance detected in WT. These results are consistent with a previous work where GRK2+/- and WT mice were fed with a HFD for a short period of time [18]. Our new data further indicate that GRK2+/- mice are protected against insulin resistance even upon the coexistence of other important intolerance-promoting factor such as age. In sum, we can now suggest that GRK2 is an important modulator of insulin tolerance even after a long-term HFD feeding, and also that lowering GRK2 levels is able to preserve insulin sensitivity even in the face of the co-occurrence of the two most important insulin resistance-prompting conditions in humans, namely age and obesity.

We also put forward that GRK2+/- mice are protected against the pathological cardiac remodeling induced by obesity. These animals do not display cardiomegaly nor cardiomyocyte hypertrophy after long-term HFD feeding while both alterations are present in control WT mice. Obesity is characterized by an inappropriate expansion and dysfunction of the adipose tissue. Even in the absence of hypertension, adiposity induces structural and functional changes in the heart through hemodynamic (volume overload) and non-hemodynamic factors such as inflammatory status, increased plasma glucose and insulin resistance, altered adipokine secretion pattern, increased levels of circulating free fatty acids, ectopic lipid deposition and lipotoxicity [22]. Independently of the specific role of GRK2 in cardiac metabolism and physiology, since decreased levels of GRK2 attenuate the “systemic” obese phenotype observed in WT, we cannot discard that the protection against obesity-induced cardiac remodeling observed in GRK2+/- mice could be promoted, at least in part, by the effect of GRK2 downregulation in other tissues helping to reduce adiposity and thus obesity. As a matter of fact, decreasing levels of GRK2 was shown to contribute to overall energy expenditure by the reduction of white adipose tissue lipogenesis and the improvement of thermogenic capacity of brown adipose tissue [23]. However, a beneficial effect for GRK2 downmodulation has been described in other types of cardiac hypertrophy, such as after thoracic aortic constriction, in genetically-based mice models of cardiac dysfunction and after myocardial infarction [24-26]. So, there are grounds to speculate that cardiac GRK2 could also be playing a direct role in modulating obesity-induced heart hypertrophy since the molecular mechanisms underlying cardiomyocyte cell growth in response to different pathological stimuli appear to have common features [27]. Consistent with this notion, we detect here an increase in cardiac GRK2 levels in WT (but not GRK2+/-) HFD-fed mice that correlates with enhanced cardiac hypertrophy. We can thus hypothesize that such increase in GRK2 amount is likely directly implicated in the cardiac remodeling observed (see below), as it was shown to be the case in cardiac hypertrophy of other etiologies [28], since GRK2 functionality has been implicated in cardiac tissue maintenance by different groups [29, 30] and its enhanced levels correlate well with different human and murine cardiac pathologies that implicate heart hypertrophy [26, 28, 31].

Notably, the HFD-induced increase in GRK2 levels in cardiac tissue appears to be an early event; in fact it was reported to be detected already after 12 weeks of HFD feeding in WT animals [17]. At this point insulin resistance is present, not only systemically [18] but also

at a molecular level in the heart as determined after an acute intravenous insulin injection [17] but no hypertrophy is yet detected in terms of the heart weight/tibial length ratio. Interestingly, a similar phenotype has been reported by several studies using genetic animal models of obesity (db/db and ob/ob) in which insulin resistance is an early event associated to the onset of the obese phenotype that occurs later (5 to 10 weeks after weaning) while left ventricular hypertrophy is only detected in much older mice [32, 33]. The precise molecular mechanism underlying the HFD-promoted increase in cardiac GRK2 levels remain to be established, although in the absence of mRNA changes, it is tempting to suggest that insulin resistance may trigger mechanisms modulating kinase stability or degradation such as those already described to control the level of this kinase in other tissues and cells [34, 35].

Hence, we could hypothesize that in our model the increase in GRK2 levels may possibly result from the metabolic remodeling that is taking place in the heart. This alteration in GRK2 dosage would subsequently affect GPCR signaling pathways, such as β-adrenergic, contributing to disturb heart physiology and lead to cardiac remodeling. The increase in GRK2 would also contribute to further impair cardiac insulin signaling and to switch cardiac catabolism towards the oxidation of lipids that are in excess because of the HFD. This could represent an initially adaptive mechanism which, after becoming chronic, results in maladaptation with detrimental consequences for the heart. In such scenario, GRK2 upregulation would be a relatively early event in the development of the metabolically-induced cardiomyopathy, as is the case for other cardiac conditions in which GRK2 levels increase in the first stages of the heart disease (see references in [31]).

Previous data from our lab detected a shift in the global transcriptional profile of the cardiac tissue promoted by GRK2 downregulation [17]. Several key genes related to cardiac functionality and pathophysiology were altered in GRK2+/- hearts even in the absence of a metabolic stress signal such as a HFD. In our current model we also detect some differential changes in the mRNA levels of several key markers of cardiac growth and functionality. An increase in BNP expression in the hearts of HFD-fed WT animals is not detected in GRK2+/- mice. Interestingly, BNP tends to be increased in hearts with depressed ventricular ejection performance or in overt heart failure, with or without hypertrophy (i.e., dilated cardiomyopathy). Likewise, increased α-Skeletal actin and decreased SERCA tend to be observed in hypertrophy models that progress to heart failure [36]. We detect here that HFD induced a marked increase in the mRNA for the Acta1 gene in WT animals, again not detected in GRK2+/- hearts. An increase in Acta1 transcription is however detected basally upon GRK2 downregulation, thus confirming previous results in which a mild, non-pathological cardiac hypertrophy develops with age in GRK2+/- mice [17]. In sum, all these results suggest that the increase in cardiac hypertrophy markers observed after HFD in WT mice is absent or non-statistically significant in GRK2+/- animals.

A last feature of HFD-induced cardiac remodeling is lipid accumulation in cardiomyocytes. Excessive lipid accumulation in the myocardium has been reported to positively correlate with the degree of obesity [37]. The increase in the percentage of fat from the diet promotes an excessive lipid exposure of the tissues that results in accumulation of triglycerides in the myocardium into cytoplasmatic lipid droplets. Inappropriate triglyceride deposition enlarges the intracellular pool of fatty acyl-CoA thereby providing substrate for oxidative and non-oxidative (for example ceramide synthesis) metabolic pathways leading to oxidative stress, cellular dysfunction and apoptosis [38]. However, such packaging of lipid excess into lipid droplets can be seen as an adaptive response to fulfill energy supply without hindering mitochondrial or cellular redox status while keeping low concentrations of lipotoxic intermediates [39]. We have observed that the amount of lipid droplets is larger in WT compared to GRK2+/- mice either after SD or HFD, so GRK2+/- mice hearts seem to be protected against excessive lipid accumulation.

As GRK2+/- mice present a leaner phenotype, the dietary fat is not accumulating in adipose tissue and is probably metabolized more efficiently than in WT mice. However, given the already mentioned role of GRK2 in fatty acid uptake and oxidation in white and brown adipose tissue [22], we cannot discard a similar direct effect of GRK2 dowmregulation in other

organs such as the heart, where fatty acid oxidation is the main source of ATP. In fact, GRK2 has been found to localize to mitochondria, whose function is critical in substrate oxidation. Some authors have described in fibroblast and endothelial cells that high levels of GRK2 promote mitochondrial biogenesis and ATP production [40] while others reported that in cardiomyocytes, increased levels of GRK2 would lead to enhanced association to mitochondria causing increased Ca^{2+}-induced opening of the mitochondrial permeability transition pore [30], a key step in cellular injury that abrogates ATP production. So, further studies are needed to evaluate the possible role of GRK2 in mitochondria in obese patients and its implication in cardiac metabolism. It is also worth noting that the amount of lipid droplets increases only in WT mice after HFD compared with their littermates fed with SD. Indeed HFD-fed WT mice showed the largest size of lipid depots. This event correlates with a worse phenotype in terms of left ventricular hypertrophy, so we would expect that the increased lipid sequestration is not only a marker but a mediator of lipotoxicity. In order to corroborate this hypothesis, it would be interesting to analyze the percentage of apoptosis and the redox status of cardiomyocytes in such conditions, a line of ongoing research in our group.

In summary, our results point to a protective role of lower levels of GRK2 upon obesity-induced cardiac remodeling and steatosis. The protection afforded by GRK2 downmodulation is apparent in the presence of concurrent relevant risk factors for insulin resistance and cardiovascular disease such as age and obesity, or after long-term HFD treatments. It is tempting to hypothesize that the increase in cardiac GRK2 protein levels triggered as a consequence of high-fat diet feeding or in different patophysiological situations, would play a central role allowing progression to maladaptive remodeling due to its unique ability to simultaneously alter cardiac β-adrenergic and insulin signaling.

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Obesity-related cardiac hypertrophy is modulated by G protein-coupled receptor kinase 2

Lucas E.[1,2], Vila-Bedmar R.[1,2], Mayor Jr. F.[1,2] and Murga C.[1,2]

1 Departamento de Biología Molecular and Centro de Biología Molecular Severo Ochoa (UAM-CSIC), Madrid, Spain.
2 Instituto de Investigación Sanitaria La Princesa, Madrid, Spain.

SUPPLEMENTAL FIGURES

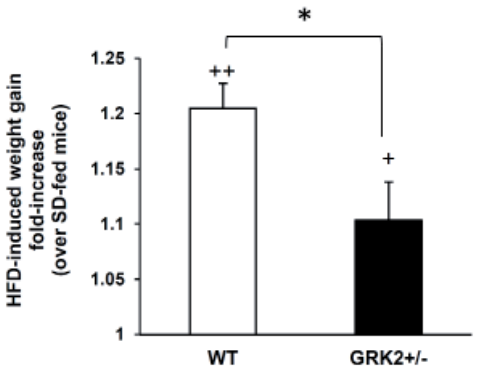

Figure S1: Weight gain induced by 33 weeks of HFD feeding in WT and GRK2+/- genotypes expressed as fold-increase over control SD-fed mice (N=5-7). Data are mean±SEM. ++p<0.01; +p<0.05 referred to SD-fed mice; *p<0.05 referred to fold increase between genotypes.

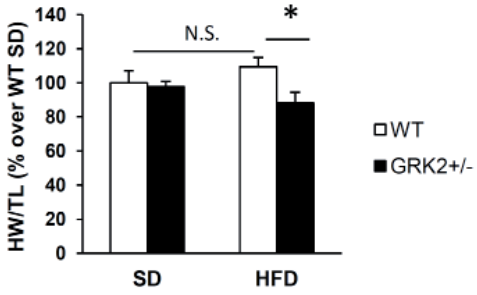

Figure S2: Heart weight to tibial length ratio after 12 weeks in HFD-fed WT and GRK2+/- mice expressed as percent change towards WT littermates fed with standard diet (N= 6-7). Data are mean±SEM. *p<0.05

DISCUSSION

As a main aim for this thesis we have tried to characterize the molecular and cellular mechanisms that underlie the impact of low GRK2 levels on key signaling pathways and phenotypic features on defined CVD pathological contexts such as vascular hypertension and obesity /insulin resistance (IR). Our results build on previous data describing the implication of GRK2 not only on the regulation of GPCR signaling, but also on non-GPCR-dependent pathways important for CVS physiology. GRK2 has thereafter arisen as an integrative sensor that may link cardiovascular disease to at least some of its comorbidities. Our data further indicate that lowering GRK2 levels help maintain some pathological features at bay and results protective for the CVS.

Along the next section we discuss the results describing the role of GRK2 on some of the signatures composing the cardiovascular disease continuum (CVDC), and on the impact of GRK2 levels in translating the influence of defined risk factors, such as obesity or hypertension, on CVDC progression.

1. GRK2 AND HYPERTENSION

Hypertension is a common condition that represents a well-known risk factor for the development of CVD. Increased peripheral vascular resistance to blood flow and vascular remodeling are common features of hypertension. Vascular tone is mainly controlled by a balance between constriction and relaxation of vascular smooth muscle cells (VSMC), but the endothelium plays a pivotal role in this regulation, affecting vascular function and remodeling [36].

1.1. GRK2 AND CELULLAR SIGNALING IN THE VASCULATURE

The physiology of both vascular cell types is finely tuned by a wide range of neurohormonal agonists. Most of them transmit their signals via GPCRs, and therefore alterations in GRK2 levels can affect both vasodilator and vasoconstrictor outputs [223-225]. Coherently, we have observed in aortas of GRK2+/- mice (*article #1*) that a partial deficiency of GRK2 enhances both acetylcholine and isoproterenol-induced relaxation, as well as phenylephrine-induced contraction. However, in our study, the increased sensitivity towards vasoconstrictor inputs upon GRK2 downregulation does not hold for all agonists. For instance, we do not observe differences in Ang II-induced contraction between GRK2+/- and WT mice aortas neither upon one acute dose nor after repeated exposure to Ang II. However, GRK2 has been described to help desensitize Ang II signals directly or indirectly in other systems [226-228]. Several aspects of GPCR pharmacodynamics could explain this apparent discrepancy. After discarding alterations in the expression of the Ang II receptors in aortic tissue (*article #1*), other parameters such as the concentration and timing of exposure to the agonist, the formation of receptor hybrids, the specific intracellular signaling cascade activated, the relative affinity of GRK2 for different GPCRs or GRK2-independent desensitization mechanisms (see below) could be playing a role [229]. In any case, at least in our experimental conditions, the partial deficiency of GRK2 is not enough to observe a direct effect on Ang II-induced contraction. However, and importantly, both chronic Ang II-induced increases in other contractile responses (such

as Phe-mediated constriction) or Ang II-triggered decreases in vasodilatory events (such as NO production) are prevented upon GRK2 downmodulation (see below).

Noteworthy, $G\alpha_s$-coupled GPCRs that mediate vasodilation are regulated by GRK2 solely via phosphorylation, whereas $G\alpha_{q/11}$-coupled receptors that mediate vasoconstriction are regulated by GRK2 by both phosphorylation-dependent and -independent mechanisms since the GRK2 RGS homology domain promotes a $G\alpha_{q/11}$-selective uncoupling of the receptor [230]. Therefore, the possibility exists that $G\alpha_s$-coupled GPCRs may display a more general enhanced sensitivity to small increases in GRK2 expression than $G\alpha_{q/11}$-coupled receptors what can contribute to the development of hypertension [224, 231, 232]. Interestingly, complete silencing of GRK2 expression in VSMC increases both vasoconstrictory and vasodilatory responses but those mediated by $G\alpha_{q/11}$-coupled receptors to a greater extent [225].

In sum, in our study (*article #1*) the partial deficiency of GRK2 upregulates different types of GPCR-mediated vasodilatory stimuli under basal conditions, while vasoconstrictory signals are not homogenously affected.

1.2. GRK2 MODULATION OF the Akt/eNOS AXIS

The main result of our study in *article #1* is the characterization of the negative role of GRK2 in the regulation of the endothelial Akt/eNOS axis in an experimental animal model of primary hypertension. Whereas Akt activation is dramatically reduced in WT mice after a chronic Ang II infusion, lower levels of GRK2 preserve the phosphorylation of Akt, which in turn would activate eNOS, leading to higher levels of NO in GRK2+/- vessels (*article #1*). A negative role of GRK2 in endothelial NO production was previously described in portal hypertension, where a direct interaction between GRK2 and Akt was reported [204], and also in a type 2 diabetes model [215]. However neither the influence of GRK2 levels in a "strong" model of systemic hypertension nor the effect of GRK2 on the Ang II-induced reduction in eNOS levels (*article #1*) had been previously reported. The mechanisms by which eNOS levels are preserved in GRK2+/- mice after a chronic Ang II insult opens an interesting field of research.

eNOS levels have been shown to be regulated by transcriptional and post-transcriptional mechanisms under different conditions [233, 234]. The RhoA/ROCK cascade downstream $G\alpha_{12/13}$, and AT1R activation decreases eNOS expression through alteration in eNOS mRNA stability [235]. In fact, statins, which have been shown to increase eNOS mRNA stability and exert protective vascular effects beyond cholesterol reduction, inhibit RhoA geranylgeranylation and ROCK activity [236]. This mechanism could be responsible, at least in part, for the decrease in eNOS levels observed particularly in WT animals after Ang II infusion.

Alternative mechanisms modulating eNOS protein degradation may also be activated in our experimental model of hypertension. eNOS is a highly sensitive substrate of calpain, a ubiquitously expressed protease whose expression is induced by Ang II [238]. Following a brief exposure to Ca^{2+}-loading, cytosolic eNOS interacts with the Hsp90

chaperone and recruits calpain forming a complex in which the synthase is almost completely resistant to digestion by the protease [239]. Since Hsp90 also interacts with GRK2 [240], one explanation for the preserved eNOS protein levels in GRK2+/- mice upon an Ang II challenge would be that in GRK2-hemizygous mice more Hsp90 protein could be available to bind eNOS, thus preventing eNOS from being degraded by calpain. Also, GIT1, a *bona fide* activator of NOS whose expression decreases during portal hypertension [241], is a GRK2-interacting protein. Therefore, in GRK2+/- endothelial cells, there might be increased activity of eNOS by increased binding to GIT, and also a better re-coupling of eNOS by a improved recruitment of activated Akt to the eNOS-Hsp90 complex [242]. Altogether, these effects would promote a partial preservation of eNOS levels, increased eNOS activation and enhanced NO bioavailability which could at least in part explain the protection against Ang II-induced vascular remodeling and hypertension observed in GRK2 hemizygous mice.

It is worth noting that, in addition to the regulation that we describe in *article #1*, it was recently reported that GRK2 and eNOS inhibit each other in murine hearts. Whereas enhanced GRK2 levels downmodulated the Akt/eNOS axis promoting a decrease in NO production, eNOS could in turn contribute to inhibit GRK2 via S-nitrosylation [205]. We cannot exclude the possibility that this regulatory loop is taking place in our experimental setting and contributing to the observed phenotype. Although we have not explored the nitrosylation state of GRK2 in our model, it is tempting to suggest that in our system increased levels of GRK2, such as those detected in Ang-II-infused vessels, could be favoring a dysregulation of this GRK2-eNOS functional interaction (increased GRK2 activity and/or decreased eNOS activity) decreasing NO bioavailability and fueling the progression of hypertension, whereas the partial deficiency of GRK2 would prevent such effects.

1.3. GRK2 IN VSMCs VERSUS ENDOTHELIUM

One of the clues for the protective role exerted by the partial deficiency of GRK2 in the CVS is that hemizygosity somehow prevents the increase in GRK2 levels above a certain threshold that is observed after a pathological insult, in this case chronic Ang II infusion. In our study, the quantification of GRK2 levels was performed in total cell lysates from aortic tissue, so we cannot elucidate if the marked increase of GRK2 levels observed in WT aortas (*article #1*), takes place in VSMCs, in endothelial cells or in both after Ang II infusion. It has been reported that GRK2 protein increases both in VSMCs [224, 225, 243] and in endothelial cells [204] during hypertension, so we would expect that the upregulation of GRK2 observed in our system occurs in both cell types, either simultaneously or as a sequential process. Thus, this pathological increase in GRK2 could affect different molecular mechanisms that should work together in the various cellular settings of the vascular system to keep blood pressure under control. In VSMCs increased levels of GRK2 would be altering GPCR signaling, preferentially downmodulating vasodilatory signals over constrictory ones [225, 232, 233](*article #1*). On the other hand, in endothelial cells increased GRK2 would affect not only GPCR signaling but also inhibit the Akt/eNOS axis, reducing NO bioavailability [204](*article #1*) required to depress agonist-induced vasoconstriction [244]. Such deleterious effects would be kept at bay

in GRK2 hemizygous animals. In addition, GRK2 has been described to modulate renal sodium transport through GPCR-dependent [245] and -independent mechanisms [246]. So GRK2 can act at different stages in the integrated system that regulates blood pressure and may exert its cardiovascular regulatory actions controlling different important signaling pathways in different cell types (Figure D1).

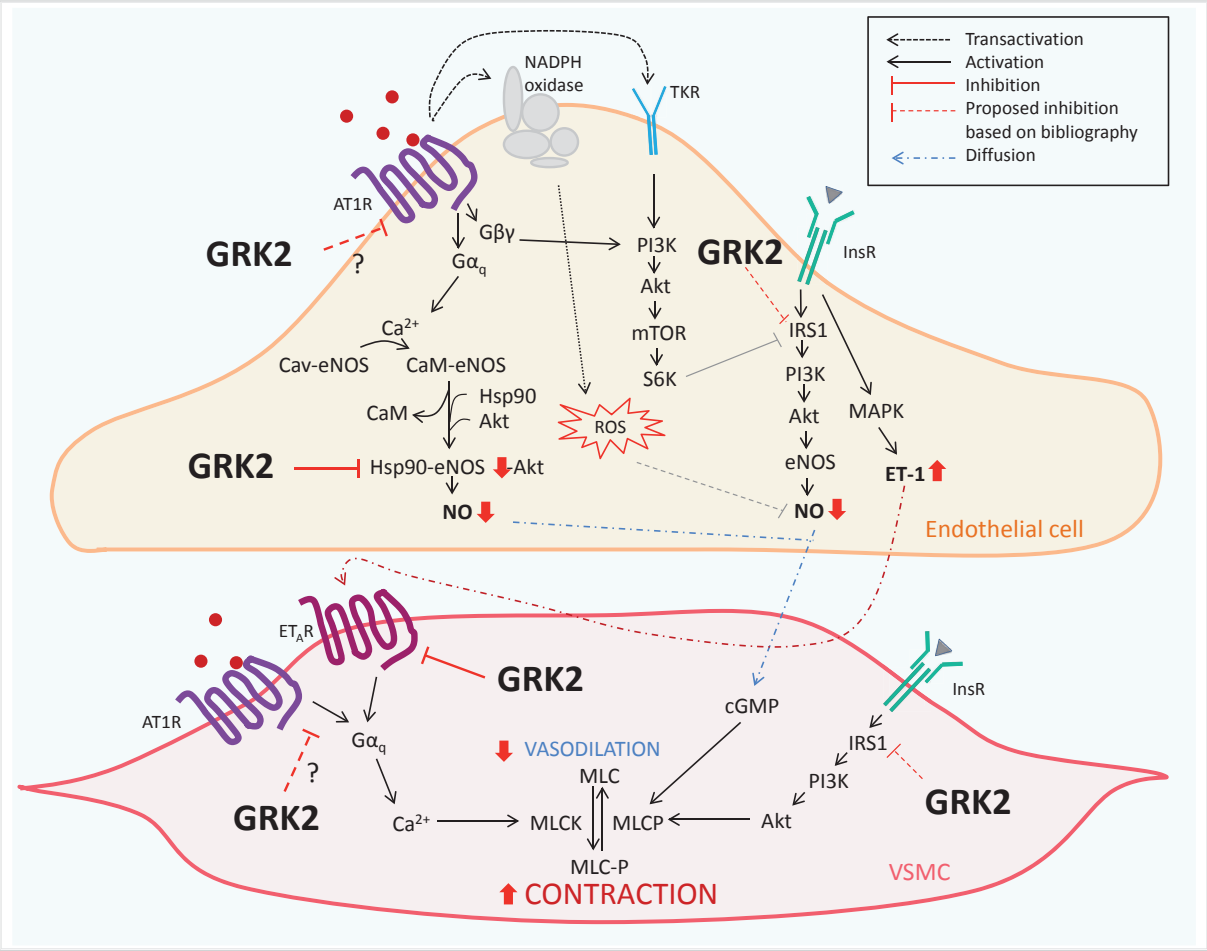

Figure D1: Role of GRK2 in endothelial and VSMCs and impact in vascular function. Independently of the role of GRK2 in GPCR desensitization, GRK2 levels could modulate the Akt/eNOS axis at several levels including direct interactions with Hsp90 or Akt. Increased endothelial GRK2 levels upon Ang II infusion or other regulatory inputs would contribute as well to lower levels of eNOS, overall decreasing NO bioavailability. Higher GRK2 dosage would also promote insulin resistance and decreased signaling by the IRS1/PI3K/Akt axis. Upon chronic Ang II infusion, this fact could be aggravated by AT1R mediated tyrosine kinase receptor (TKR) transmodulation and enhanced ROS formation via NADPH oxidase, further unbalancing NO and ET-1 production and contributing to endothelial dysfunction. In VSMCs, decreased NO bioavailability, enhanced Ang II and ET-1 binding to their receptors, and impaired insulin sensitivity, would favor increased contraction, hypertension and vascular remodeling. These effects would be prevented in animals with lower levels of GRK2 (see text for detailed discussion).
Cav, caveolin; CaM, calmodulin; MLC, myosin light chain; MLCP, MLC phosphatase; MLCK, MLC kinase. cGMP, guanylate cyclase. Red thick arrows reflect consequences of increased levels of GRK2.

1.4. GRK2, ENDOTHELIAL DYSFUNCTION AND INSULIN RESISTANCE

Endothelial dysfunction is one manifestation of the many changes induced in the arterial wall by the metabolic abnormalities accompanying diabetes and insulin resistance

(IR). Under IR conditions, endothelium-dependent vasodilator function is compromised because of altered production of both vasodilator and vasoconstrictor substances, in particular decreased bioavailability of NO, decreased production of prostacyclin as well as increased production of thromboxane, other cyclooxygenase-dependent vasoconstrictors and endothelin [247].

Adult 9 month-old WT mice were described to exhibit higher blood glucose and insulin levels at baseline and also decreased systemic glucose and insulin tolerance than their GRK2+/- littermates [170]. This pre-diabetic state correlates with impaired insulin cascade activation in several tissues such as adipose tissue, liver, muscle [170] and heart (*article #3*). So it is tempting to assume that, in the adult WT mice used in our study, a certain degree of vascular IR exists that may contribute/predispose to the development of endothelial dysfunction. Endothelial IR selectively affects the PI3K/Akt/eNOS axis downstream the insulin receptor [248] thus reducing the production of NO in favor of ET-1 and enhancing vasoconstriction. Therefore, GRK2+/- mice could be protected from hypertension, at least in part, because their increased sensitivity to insulin. Moreover, insulin can activate and upregulate eNOS gene expression [249] what correlates with the tendency of eNOS levels to be higher in GRK2+/- than in WT adult mice in the absence of Ang II (*article #1*). In addition, this mechanism could contribute to the increased preservation of eNOS levels in GRK2+/- aortas after Ang II infusion (*article #1*). In conclusion, GRK2 may exert its cardio-regulatory actions as a nodal point linking different signaling pathways that connect cardiovascular physiology and metabolism, with insulin signaling regulation playing a central role (Figure D1).

2. GRK2 AND CARDIAC INSULIN SIGNALING

Cardiac IR is a metabolic and functional disorder with clear detrimental effects for the heart´s physiology. Major factors contributing to the development of cardiac IR are oxidative stress, hyperglycemia, hyperlipidemia, dysregulated secretion of adipokines/cytokines and inappropriate activation of renin-angiotensin II-aldosterone system (RAAS) and the sympathetic nervous system [250]. Some of these features are representative of aging and overnutrition, situations where we have described that lower levels of GRK2 prevent the development cardiac IR (*article #3*).

2.1. GRK2 AS A NEGATIVE MODULATOR OF CARDIAC INSULIN SIGNALING

A relevant contribution of our work is that GRK2, previously reported to negatively regulate the insulin receptor cascade in different tissues such as liver, adipose and muscle (reviewed in *article #2*) is also an important modulator of insulin-mediated signaling in cardiac tissue (*article #3*).

2.1.1. AGE-INDUCED INSULIN RESISTANCE

Insulin signaling in the heart is essential for growth and development, and for normal homeostasis of glucose, fat, and protein metabolism, so it is tightly regulated by

a complex network of phosphorylation/dephosphorylation mechanisms, and by negative feedback loops [251]. In other tissues [170], there is a certain impairment in the insulin cascade activation in adult WT animals, while insulin signaling is completely preserved in GRK2+/- mice. In *article #3* we report significant differences upon acute insulin stimulation in adult 9 month-old mice GRK2+/- hearts compared to their WT littermates. The rapid insulin cascade activation is enhanced in adult GRK2+/- mice and also quickly shuts down as can be observed when comparing 3 and 5 minutes of stimulation (*article #3*). Interestingly, an effective downmodulation of insulin signaling after the activation of the cascade is critical to preserve the cardioprotective role of insulin. Indeed, excessive insulin signaling due to alterations in its regulatory mechanisms of control, as happens during hyperinsulinemia, is detrimental for the cardiac tissue. The chronic activation of the cascade can promote, among other effects, a pathological hypertrophic response and systolic dysfunction [252]. So, the lack of a basal chronic activation of the insulin cascade in GRK2 hemizygous animals, together with an enhanced acute signaling and normal shutdown, could serve to preserve the beneficial effects of insulin signaling in the absence of the detrimental cardiac consequences of sustained insulin cascade activation.

Notably, the increased sensitivity to insulin observed in cardiac tissue of GRK2+/- mice selectively affects the PI3K/Akt axis, since no changes in the activation of the MAPK axis were observed in the heart compared to WT mice (*article #3*). This correlates with what was described in vessels [248], so we predict that GRK2 modulation could have as a consequence a "biased" insulin resistance in several important tissues of the CVS. This "biased IR" imposed by altered GRK2 levels would be characterized by an specific downregulation of the IRS1/PI3K/Akt branch downstream the insulin pathway upon enhanced GRK2 expression in pathological settings, whereas it would be key to explain the cardioprotective role exerted by a partial deficiency of GRK2 (Figure D2, see below).

2.1.2. DIET-INDUCED INSULIN RESISTANCE. THE ROLE OF GRK2-IRS1 COMPLEXES

Not only age-induced IR but also diet-induced IR is avoided in GRK2+/- cardiac tissue. We observed that 12 weeks of HFD are enough to promote cardiac IR in young WT 4 month-old mice, while insulin signaling sensitivity was completely preserved in GRK2 hemizygous mice (*article #3*). Most interestingly, we found increased GRK2 levels in the hearts of mice fed with HFD and in adult ob/ob cardiac tissue in both animal models of IR. Our study has been the first to unveil the role of GRK2 in diet-induced IR in cardiac tissue.

We find that in these models, increased levels of GRK2 correlate with higher amounts of IRS co-immunoprecipitated with GRK2 (*article #3*), as so happens in other cell types (discussed in *articles #2* and *#3*). This is consistent with GRK2 being an important negative modulator of insulin signaling that could serve to link cardiac metabolism and functionality. Nevertheless, there are some aspects of the molecular mechanisms underlying the formation of GRK2-IRS1 complexes and their functional consequences that are still unsolved.

It has been described that GRK2-IRS1 complex formation promotes IRS1 phosphorylation in Serine 307 by GRK2 ([253]; *article #2* and references herein). Such

phosphorylation event has been reported to disrupt IRS1 binding to the insulin receptor and to promote IRS1 proteasomal degradation [254] under different IR conditions. However, we were unable to detect neither IRS1 phosphorylation in Ser307 nor changes in the total levels of IRS1 in our animal model. So, we postulate that IRS1 sequestration by GRK2 would be sufficient to impair signaling by hindering PI3K/Akt axis activation. However, it is important to highlight that, although the negative modulation of insulin signaling by GRK2 seems to be a general event that takes place in several cell types, the molecular mechanisms participating in each specific tissue (IRS1 phosphorylation in Serine 307; formation of the GRK2/IRS1 complex) may vary depending on the cellular context.

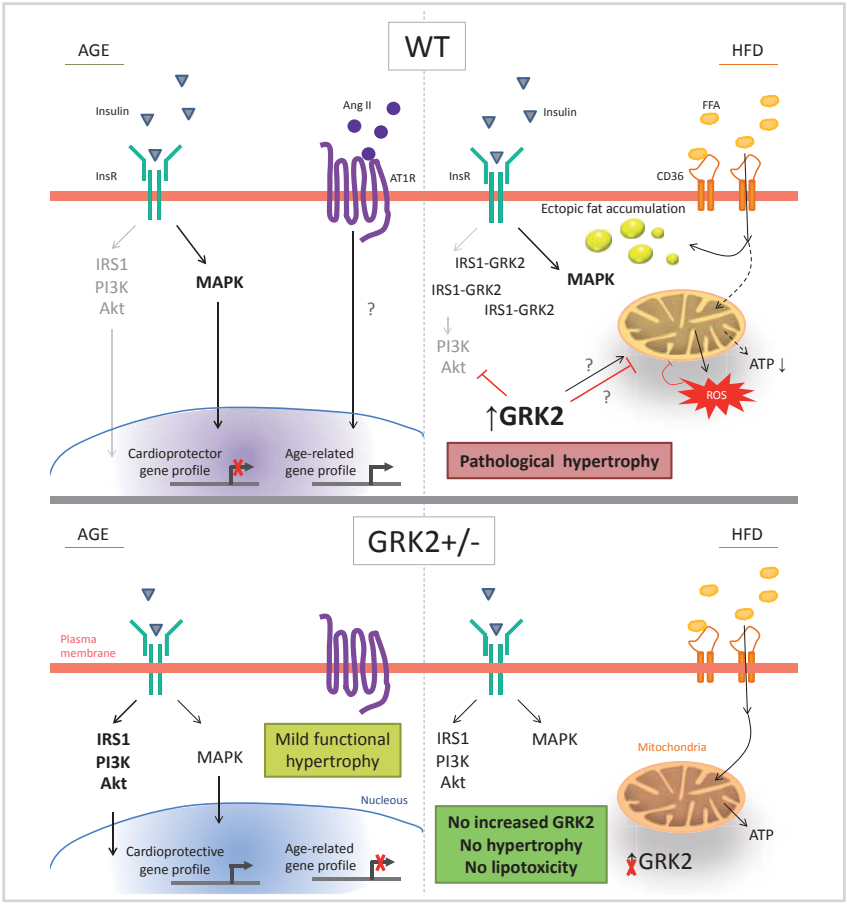

Figure D2: Effects of modulating GRK2 levels in age and long-term HFD feeding-related cardiac phenotypes. Adult WT mice display cardiac insulin resistance what may promote, together with other age-related alterations, changes in the gene expression profile related to pathological remodeling and diabetes, whereas a partial deficiency of GRK2 (GRK2+/- mice) protects from age-related alterations such as insulin resistance. The cardioprotective role of lowering GRK2 translates into the upregulation of genes more related to physiological hypertrophy and response to exercise, and, at a macromolecular level, into mild cardiac hypertrophy that seems to be physiological. On the other hand, a HFD-induced increase in GRK2 levels aggravates insulin resistance by the formation of GRK2-IRS1 complexes and may alter mitochondrial function leading to increased ROS production, decreased ATP production and accumulation of intracellular fat from the diet. Altogether these features of lipotoxicity would promote cardiac dysfunction and pathological hypertrophy. In GRK2+/- hearts, GRK2 levels do not increase above the pathological threshold, so they are protected against the deleterious effects of HFD in cardiomyocytes (see text for detailed discussion).
InsR, Insulin receptor; FFA, free fatty acids. Black arrows reflect activated pathways whereas grey arrows mean downmodulated pathways. Dotted arrows reflect impaired process and red lines, inhibition.

2.2. GRK2 DOWNMODULATION, AGING AND GENE REPROGRAMMING

Apart from the evident preservation of insulin sensitivity observed in GRK2 hemizygous mice hearts, these animals also show increased induction of genes that exert cardioprotective actions while the expression of genes related to pathological heart hypertrophy were constitutively repressed (*article #3*). Notably, such differences were only observed in adult GRK2+/- mice, and not in younger animals (4 months old) suggesting that the effects of GRK2 dosage on cardiac gene expression become apparent only in the face of age-related changes. In fact, there are several examples of genes typically upregulated with age such as Timp4 [255] or Nppa [256] that are downregulated in adult GRK2 hemizygous mice.

Several signaling pathways have been shown to be involved in cardiac aging such as the insulin/IGF/PI3K and the renin-angiotensin II systems [257]. As described in *article #3*, the preserved cardiac insulin sensitivity in GRK2+/- mice could explain the upregulation of several genes reportedly modulated by insulin observed in this genotype (Nr4a1, Tcfl2, Rrad, Aqp7, Cebpb, Ppargc1b, Egr1). On the other hand, genes found to be downregulated in GRK2+/- mice are upregulated in cardiac tissue by prorenin (Ptgds) or Ang II (Nppa and Zbtb16) [258, 259].

It is also worth mentioning here that the age-induced changes in gene expression controlled by the insulin and the angiotensin pathways are further related to cardiac remodeling and hypertrophy of a pathological kind [257]. On the contrary, the gene expression profile of GRK2 hemizygous is of a cardioprotective nature, what could be due, at least in part, to the preserved insulin sensitivity (*article #3*) and the increased NO bioavailability (*article #1*), both well-established cardioprotective systems (Figure D2).

3. GRK2 AND OBESITY-INDUCED CARDIAC REMODELING

To deepen on the cardioprotective role of GRK2, we performed a study to evaluate if lower levels of GRK2 could protect the heart against HFD-induced maladaptive remodeling of the cardiac tissue (*article #4*). This approach allows us to determine if the mild hypertrophy observed with age in adult GRK2+/- mice (*article #3*) predisposes the heart to maladaptive remodeling upon a pathological insult such as HFD, or, on the contrary, if it can help prevent or decrease pathological cardiac remodeling.

3.1. GRK2, OBESITY AND LIPOTOXICITY

One of the clues to discuss the results of *article #4* is to determine the influence of GRK2 in the modulation of systemic processes vs the control of cardiac-specific ones. GRK2+/- mice seem to be protected against diet-induced obesity as shown by the ameliorated diet-induced body weight gain and the preserved systemic insulin tolerance (*article #4*). However, we observed that although the weight of epididymal white adipose tissue (WAT) increases in both genotypes after a long term HFD, there are no differences in WAT weight between WT and GRK2+/- mice (data not shown) as opposed to what we find after a short-term HFD feeding. One plausible explanation for the differences

in body weight and insulin sensitivity we observe between genotypes could be that fat accumulation and lipotoxicity are taking place ectopically in WT mice, thus contributing to the increase in overall body weight and to peripheral IR independently of changes in adipose tissue. In this case, GRK2+/- mice may, by some mechanism, be accumulating less ectopic fat thus resulting in a protection against these pathological events. In line with this hypothesis, we observe an increased amount of intracellular lipid droplets in cardiac tissue (*article #4*) and increased liver weight in WT HFD-fed mice that could be due to liver steatosis (data not shown). Fat accumulation in heart and liver weight after a HFD was less pronounced in GRK2+/- mice. Although there are no reports to our knowledge describing a role for GRK2 in ectopic fat accumulation, we believe our results suggest there is a direct or indirect implication of GRK2 in this process. For instance, the decreased amount of intracellular lipid droplets observed in GRK2+/- cardiomyocytes, either after HFD or SD, could indicate that FFAs are not entering the cell in excess or are being more efficiently metabolized. It will be thus interesting to study the levels and dynamics of CD36, the main fatty acid transporter, and of lipoprotein lipase, required for the uptake of FFAs from lipoprotein triglycerides [260] in such conditions. However, if the uptake of FFA from the blood flow is limited in GRK2+/- mice, the excess FFA from the HFD must be undergoing a different fate. As discussed in *article #4*, one possible explanation would be that the excess FFA are being oxidized in the brown adipose tissue by means of the better thermogenic capacity found in adult GRK2 hemizygous mice [169]. Interestingly, the possibility exists that this relationship between the improved oxidative phenotype and lower levels of GRK2 described for adipose tissue could be extrapolated to peripheral tissues such as muscle, liver, or heart. If this is the case, lowering GRK2 could result beneficial to avoid ectopic fat accumulation and lipotoxicity. This possibility is currently being investigated in our laboratory.

3.2. GRK2 AND MITOCHONDRIA

A key determinant of metabolic efficiency is an improved mitochondrial function. As mentioned above, several data suggest that GRK2 dosage could be playing a role on the control of mitochondrial fatty acid oxidation (FAO) either in an indirect or in a direct manner. As an example of the former, the increased NO bioavailability described in *article #1* for GRK2+/- adult mice could be linked to increased mitochondrial biogenesis and enhanced coupled respiration and ATP production [261] (see below).

In addition, a direct effect of GRK2 on mitochondrial function could also be envisaged. The mitochondrial localization of a population of GRK2 has been described [151, 152] that suggests a potential role for GRK2 in the regulation of energy metabolism. However, to date there are only a few and apparently contradictory reports on the role of GRK2 in mitochondria. It seems clear that GRK2 can be found inside the mitochondria in resting conditions [151, 152] and that GRK2 increases its mitochondrial localization upon stressful stimuli such as inflammation or ischemia/reperfusion [152, 262, 263] in different cell types. Nevertheless, the role that GRK2 exerts in mitochondria is more controversial. While some authors described that GRK2 enhances mitochondrial biogenesis thus leading to an increase in cellular ATP content [151], others suggest that increased GRK2 promotes mitochondrial-based apoptosis [152]. Further study is required to determine if

in our model (upon the stress of a fatty diet) GRK2 is detrimental or advantageous, and the precise role that GRK2 could play on the regulation of metabolic plasticity and, in particular, on the control of mitochondrial fatty acid oxidation (FAO).

The most plausible hypothesis in our model would be that cardiac IR (*article #3*) in the face of hyperinsulinemic conditions (characteristic of overt T2DM) would be promoting an increased ERK activation that would lead to GRK2 phosphorylation on Ser670 in WT mice. Phosphorylated GRK2 would then interact with Hsp90 and together translocate into the mitochondria where GRK2 would promote an increased Ca^{2+}-induced opening of the mitochondrial permeability transition pore [264]. This would in turn result in proton ''leak'' and dissipation of the membrane potential. Such dysfunctional mitochondria would not be able to sustain enough levels of FAO to consume the excess FFA incoming from the diet hence precipitating the accumulation of FFA in intracellular lipid droplets. Hemizygous mice, on the contrary, do not present hyperinsulinemia, and GRK2 would neither be phosphorylated by ERK nor translocated inside mitochondria. Mitochondrial function would be preserved and FAO would be active enough to burn the excess fat thus preventing FFA accumulation in lipid droplets.

However, the hypothesis of a positive role of GRK2 on mitochondrial biogenesis and function would also fit with our results since, as part of an adaptive mechanism, GRK2 could translocate to the mitochondria to promote an enhanced FAO, in an attempt to accommodate the excess of incoming fat. Similar to other adaptive mechanisms, the effect could be helpful at the very beginning, but, eventually, become detrimental for the physiology of the cell when the stimulus that promoted it becomes chronic. An exacerbated increase in the rate of FAO could eventually lead to its own downregulation due the increase in myocardial oxygen consumption, the uncoupling of the mitochondria and/or an enhanced production of ROS [31].

In sum, decreased levels of GRK2 may protect from lipotoxicity and preserve an insulin-sensitive state that prevents both the metabolic-induced perturbations and the pathological hypertrophy caused in cardiac tissue by a high fat diet feeding (*article #4* and Figure D2).

3.3. ANTIOBESOGENIC ROLE OF NO

In animals, energy metabolism is regulated both at a cellular and a systemic level involving inter-organ cooperation. This is particularly important for the function of physiological levels of NO that stimulate blood flow, thereby increasing the supply of fatty acids, glucose, and oxygen to tissues and, therefore, the mitochondrial oxidation of these substrates [265].

It is estimated that physiological levels of NO stimulate glucose uptake and oxidation by 25–40%, as well as fatty acid uptake and oxidation by 30–40% in insulin-sensitive tissues (muscle, heart, liver, and adipose tissue) [266, 267]. The underlying mechanisms may involve multiple cGMP-dependent pathways in insulin target tissues [265]. First, NO stimulates the phosphorylation of AMPK [268] what results in increased

transport of glucose, better import of long-chain fatty acid transport from the cytosol into the mitochondrion and decreased expression of genes related to lipogenesis and gluconeogenesis [269]. Second, NO increases the phosphorylation of hormone-sensitive lipase (HSL) and perilipins via activation of cGMP-dependent protein kinase (PKG). When both HSL and perilipins are phosphorylated, HSL interacts with perilipins and this association triggers the translocation of HSL from the cytosolic compartment to the surface of lipid droplets. This initiates lipolysis thus precluding cardiac steatosis [270-272]. Third, NO activates expression of PGC-1α, which is a key regulator of oxidative phosphorylation in mitochondria [273]. Long-term effects of physiological NO levels include beneficial increases in mitochondrial biogenesis (de novo formation of metabolically active mitochondria) [274].

All these mechanisms may help explain the phenotype observed in GRK2+/- mice after a HFD. Given that GRK2+/- exhibit higher NO bioavailability either under basal or after pathological conditions (Ang II infusion-induced hypertension, article #1), it is plausible that NO actions on cardiac mitochondrial metabolism can participate in the protection against diet-induced obesity observed in GRK2 hemizygous mice.

4. PATHOLOGICAL CHANGES IN GRK2 LEVELS: FUNCTIONAL IMPACT AND MECHANISMS INVOLVED

Emerging evidence indicate than an increase in the levels of catecholamines [275] and/or angiotensin (*article #1*) and/or dietary fat (*article #3* and *#4*), results in upregulated GRK2 levels what may help fuel additional maladaptive mechanisms. Our data suggest that cardiac or vascular GRK2 levels could act as an integrative sensor of a variety of pathological inputs in the CVS and serve as a point of convergence of different noxious stimuli such as a deregulated GPCR or insulin cascades. If the levels of GRK2 increase in the heart, and this increase is persistent in time, the sustained β-AR desensitization would fuel a detrimental cycle where the adrenal glands will try to compensate the lower β-adrenergic output by increasing catecholamine secretion. This in turn will further upregulate GRK2 levels in the cardiac tissue [276], thus triggering an impairment of cardiac insulin signaling and causing a metabolic shift towards fatty acid uptake and oxidation (discussed in *article #3*) further hampering insulin signaling. Together, these events would eventually bring about maladaptive changes in global gene expression patterns. Thus, altered GRK2 levels may trigger a "downhill from here" event as it fuels several detrimental cycles that inexorably lead to maladaptive cardiac remodeling and heart failure. In this context, the alteration of GRK2 levels induced by dietary fat would be part of the metabolic remodeling of the heart, an early event that initially may be adaptive, but that, when it becomes chronic, irreversibly unbalances the homeostasis of the cardiac system. Overall, our data put forward GRK2 as a new molecular link among aging, insulin resistance/obesity and cardiovascular co-morbidities.

An important question that remains unsolved is what is/are the molecular mechanism(s) that promote(s) the increase in GRK2 levels observed in our animal models

of hypertension and obesity, and also whether this increase is a cause or a consequence of the pathology.

Although not analyzed in the Ang II model of hypertension, no changes in GRK2 transcription after HFD were detected in the diet-induced obesity model (*article #4*) suggesting that, as so happens in other animal models of pathology (reviewed in [199]) alterations in GRK2 levels are governed by post-transcriptional mechanisms. One such mechanism may implicate Hsp90. Hsp90 was described to protect GRK2 from degradation thus promoting its accumulation [240]. However the precise events triggering GRK2/Hsp90 interaction are unknown. In addition, Hsp90 stabilization is complex including post-translational controls [277]. Moreover, to our knowledge, there are no evidences of alterations in Hsp90 levels under pathological conditions such as hypertension and obesity, and an upregulation of Hsp90 has been described in the hearts of type I diabetic rats [278, 279]. Therefore, the implication of the Hsp90 chaperone in GRK2 upregulation upon pathological insults remains a subject for future investigation.

Other possible explanation of increased GRK2 levels is an impaired degradation by a decreased interaction with Mdm2. Mdm2 is an E3 ubiquitin ligase implicated in protein degradation by the proteasome. It has been described that, in a tumoral context, Akt activation downstream IGF-I receptor blocks Mdm2-dependent GRK2 degradation through the phosphorylation of Mdm2 [190]. However, we believe this mechanism is not taking place in our models for two reasons: i) Akt activation is decreased by the establishment of an insulin-resistant state, and ii) active Akt would correlate with lower levels, and not with an upregulation, of GRK2. In any case, we cannot discard the possibility that alterations of the Mdm2/GRK2 interaction could be playing a role in the final steady-state levels of GRK2 observed under diseased conditions.

On the other hand, GRK2 downmodulation also occurs in several pathological and, mostly, physiological conditions. Several studies have demonstrated that chronic exercise promotes a decrease of GRK2 levels/activity in the CVS [280] that could exert a protective role against CVD. Moreover, exercise improves vascular insulin sensitivity in spontaneously hypertensive rats via downregulation of vascular GRK2, what may help limit the progression of hypertension [281], suggesting that at least part of the cardioprotection achieved through exercise could be related to GRK2 downregulation. A similar effect was described in a left ventricular assisted device study in humans where the decrease in GRK2 paralleled the amelioration of heart failure [282]. Interestingly, irisin, a myokine secreted by the muscle upon exercise, partially prevents obesity and T2D, an effect that mirrors that of reduced GRK2 levels [283]. It would be thus of interest to explore whether irisin or other myokines released from the muscle upon exercise could somehow regulate GRK2 levels what remains a subject for future research.

In sum, it appears that GRK2 levels are increased or reduced in response to the worsening or amelioration of different pathologies, and therefore they can potentially act as an intracellular sensor of the adaptive or maladaptive responses of the cell to various detrimental insults. However it is hard to determine if higher GRK2 levels are the cause or the consequence of a pathological insult. Changes in the levels of GRK2 over

a physiological threshold appear to fuel detrimental loops where pathology worsens in parallel to GRK2 upregulation. So, the possibility exists that GRK2 increases could be at the same time one of the causes and also one of the consequences of a given pathology depending on the degree of development and the stage of the disease.

5. PHYSIOLOGICAL IMPLICATIONS OF LOWERING GRK2 LEVELS AND THERAPEUTIC OPPORTUNITIES

GRK2 is an important modulator of cellular signaling in different cell types and under various cellular contexts. Our studies have contributed to build a new concept about the role of GRK2 in the cell: that of a "stress-sensing" protein acutely upregulated or mobilized by pathological insults such as chronic Ang II infusion (*article #1*) or excessive fat from the diet (*article #3* and *#4*). We have also shown that GRK2 dosage promotes gene reprogramming with age that may have consequences at a tissue level (*article #3*).

GRK2 is very important for physiology, and, indeed, gene ablation of GRK2 is embryonic lethal [159]. As mentioned above, its levels decrease with exercise [280], and this seems to be beneficial, but if they are completely deleted in the CVS, a GRK2 reduction becomes deleterious for the vessels [224, 226], although this is not the case for cardiac tissue [166]. In fact, when cardiomyocyte GRK2 expression was ablated after myocardial infarction, cardiac remodeling and function is partially preserved although the unharmed phenotype is not completely rescued [166]. Of note, this study was performed in young animals, but the effects of deleting GRK2 in adult animals where pathways such as β-AR, Ang II or even insulin are already altered may have effects in the heart that have not been explored. On the other hand, an increase in GRK2 levels also results damaging for the CVS as has been extensively demonstrated ([284]; *article #1, #3* and *#4*). So, a physiological window may exist for GRK2 levels where an upregulation over basal may result pathological but where controlled downregulations inside a physiological range may be beneficial. Thus, there might be a pathological threshold beyond which a further elevation in GRK2 levels could initiate its contribution to disease development or to the aggravation of the pathology.

For all these reasons, the therapeutic opportunities of decreasing GRK2 activity/levels can become certainly appealing. Several groups are trying to develop inhibitors of GRK2 kinase activity, as described in the Introduction, and although to date there are no selective small molecule inhibitors with proper pharmacological properties, it seems that the development of a good pharmacological inhibitor of GRK2 kinase activity is closer every day. However, GRK2 also elicits kinase-independent actions as a scaffold or as a signaling hub that would be very difficult to abrogate or control by small molecule-based approaches. In those pathologies where GRK2 functions are more related to its scaffolding abilities or to its interacting partners, strategies aimed at decreasing GRK2 levels by different means such as exercise, increased degradation or downregulation via neuro-hormonal approaches could constitute a better alternative.

Other aspect that is important from a therapeutic point of view is whether lowering GRK2 activity and/or levels would rescue an individual from an already established pathological phenotype. As mentioned in the Introduction, the CVDC starts as a remodeling processes of the myocardium and vasculature in response to a range of potentially noxious stimuli but, when these changes persist, it progresses to structural alterations that become self-perpetuating and pathological [8]. It may appear that, as derived from inducible models of GRK2 deletion after myocardial infarction [166], a treatment to downmodulate GRK2 once the pathological situation is overt would not fully rescue the initial condition, but it can nevertheless partially improve the phenotype. Unpublished results from our laboratory using tamoxifen-inducible GRK2 KO mice also indicate that a deletion in GRK2 after HFD-induced systemic IR is already established can help ameliorate this condition ([285], submitted). Finally, the use of beta blockers for the treatment of heart failure has additional benefits to the use of βARKct transgenesis alone [147, 286]. Thus, GRK2 could have a role not only in the prevention but also in the treatment of certain pathologies, and a potential for the combination of GRK2 inhibitors with other therapeutic strategies represents a field of promising new research.

CONCLUSIONES/CONCLUSIONS

1.-Los ratones adultos (9-10 meses de edad) con menores niveles de GRK2 (GRK2+/-) muestran una mayor sensibilidad ante algunas señales vasoconstrictoras, mientras que son más sensibles a la mayoría de estímulos vasodilatadores, en parte gracias a un aumento de la biodisponibilidad de óxido nítrico.

2.- Tras una estimulación crónica con angiotensina II (Ang II), los ratones GRK2+/- adultos conservan la activación de la cascada de Akt, mayores niveles de la enzima eNOS y la biodisponibilidad de óxido nítrico preservada en comparación con sus hermanos de camada de genotipo silvestre. Los ratones GRK2+/- muestran también una estructura vascular y un comportamiento mecánico de los vasos menos deteriorado después del tratamiento con Ang II. Estas características protegen a los animales hemicigotos para GRK2 del desarrollo de hipertensión severa y remodelado vascular patológico inducido por Ang II.

3.- La reducción de GRK2 en los ratones GRK2+/- adultos promueve una mayor sensibilidad a la insulina en corazón, lo que se correlaciona con un perfil de expresión génica cardioprotector y una hipertrofia fisiológica leve. Por el contrario, los niveles de GRK2 aumentan en el tejido cardiaco de modelos animales de resistencia sistémica a la insulina, tales como animales alimentados con dieta alta en grasa o ratones ob/ob. Estos mayores niveles cardiacos de GRK2 parecen desencadenar la alteración en la sensibilidad a la insulina cardiaca por mecanismos que implican una mayor formación de complejos GRK2/IRS1.

4.- Los ratones GRK2+/- adultos muestran un fenotipo más delgado y están protegidos contra la hipertrofia cardíaca patológica y la acumulación ectópica de grasa que tiene lugar en los corazones de los animales de tipo silvestre después de un largo periodo (33 semanas) de alimentación con una dieta alta en grasa.

CONCLUSIÓN GENERAL:

Nuestros resultados presentan a GRK2 como un sensor integrador de estímulos patológicos de diferente etiología. Unos niveles elevados de catecolaminas/angiotensina en estados hipertensivos, o situaciones asociadas a resistencia sistémica a insulina (obesidad/alto contenido de grasa en la dieta) convergen en promover un aumento en los niveles de GRK2 en tejido cardiaco y/o vascular. Teniendo en cuenta el papel de esta proteína como nodo central de las cascadas de señalización de GPCRs y de insulina, estos mayores niveles/funcionalidad de GRK2 favorecerían la progresión hacia procesos de maladaptación patológica, remodelación cardíaca/vascular y disfunción. Por el contrario, el mantenimiento de los niveles de GRK2 por debajo de un cierto umbral sería beneficioso para el metabolismo y la función cardíaca, así como para mantener el tono vascular y la sensibilidad a la insulina sistémica. Por lo tanto GRK2 emerge como una diana terapéutica potencialmente relevante en la insuficiencia cardíaca, en particular cuando comorbilidades tales como resistencia a la insulina, diabetes y la obesidad están presentes.

1.-Adult mice (9 months old) with lower levels of GRK2 (GRK2+/-) show increased sensitivity to certain vasoconstrictor signals, whereas GRK2+/– arteries are more sensitive to most vasodilator stimuli in part by means of an increased bioavailability of nitric oxide.

2.- Upon chronic Angiotensin II (Ang II) challenge, adult GRK2+/- mice display a preserved activation of the Akt cascade, higher levels of the eNOS enzyme, and preserved nitric oxide bioavailability compared to wild type littermates. GRK2+/- mice also show a less deteriorated vascular structure and mechanics after an Ang II treatment. These features protect GRK2 hemizygous animals from developing severe Ang II-induced hypertension and pathological vascular remodeling.

3.- The lower GRK2 dosage in GRK2+/- mice promotes enhanced insulin sensitivity in adult hearts what correlates with a cardioprotective gene expression profile and mild physiological hypertrophy. Conversely, GRK2 levels increase in cardiac tissue in animal models of systemic insulin resistance, such as high fat diet-fed animals or ob/ob mice. Such increased cardiac GRK2 levels appear to trigger impaired cardiac insulin sensitivity by mechanisms involving enhanced formation of GRK2/IRS1 complexes.

4.- Adult GRK2 +/- mice display a leaner phenotype and are protected against the pathological cardiac hypertrophy and against the ectopic lipid accumulation that occurs in the hearts of wild type littermates after a long term (33 weeks) high-fat diet feeding.

OVERALL CONCLUSION:

Our results put forward GRK2 as an integrative sensor of pathological inputs of different etiology. Increased levels of catecholamines/angiotensin in hypertensive conditions, or systemic insulin-resistance-associated situations (obesity/high dietary fat) would converge on promoting enhanced GRK2 expression in cardiac and/or vascular tissues. Given the role of this protein as a central node of both GPCRs and insulin signaling cascades, such increased GRK2 levels/functionality would play a key role in allowing progression to pathological maladaptation, cardiac and vascular remodeling and dysfunction. On the contrary, maintaining GRK2 levels below a certain threshold would be beneficial for cardiac metabolism and function, and also for maintaining vascular tone and systemic insulin sensitivity, thus emerging as a potentially relevant therapeutic target in heart failure, particularly when co-morbidities such as insulin resistance, diabetes and obesity are present.

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APPENDIX

The CD-ROM contains:

- This thesis in PDF format
- The articles already published or submitted by the author
- The Curriculum Vitae of the author